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Integrated Role of Intramuscular Inflammation, Dietary Protein, and Testosterone Supplementation in the Regulation of Skeletal Muscle Mass during Muscle Wasting Conditions

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Skeletal muscle atrophy occurs with disuse after musculoskeletal injury, and during periods of sustained energy deficit. Manipulating dietary protein intake or supplementing with testosterone under muscle wasting conditions may protect muscle mass and/or function by increasing muscle protein synthesis, attenuating muscle protein breakdown, and enhancing myogenesis either directly or via upstream modulation of inflammatory signaling. The protective effect of dietary protein was evaluated in Study 1. Vastus lateralis muscle collected perioperatively from anterior cruciate ligament (ACL) reconstruction patients assigned to an optimal (OP: 2.0 $g \cdot kg^{-1} \cdot d^{-1}$, n =3) or adequate (AP: 1.0 $g \cdot kg^{-1} \cdot d^{-1}$, n =2) protein diet for two weeks prior to surgery was analyzed for intracellular markers of myogenesis, inflammation, and protein turnover. Thigh circumference and strength were measured before surgery and 2, 4, and 8 weeks postoperatively. Testosterone-mediated intracellular signaling regulating muscle mass was determined in Study 2 before and after a 28-day, severe diet- and exercise-induced energy deficit with weekly supplementation of 200 mg of testosterone enanthate (TEST; n=10) or a placebo (PLA; n=10) at baseline, 60 minutes after a bout of cycle ergometry, and 6 hours post-exercise. A protein-containing meal was consumed following the second biopsy.

Higher protein intake before surgery did not influence perioperative markers of inflammation, myogenesis, or protein synthesis. Gene expression of the proteolytic marker

muscle ring finger 1 (MuRF1) was greater in AP versus OP individuals at the time of surgery. Postoperative thigh circumference at week 2, 4, and 8 relative to baseline was associated with perioperative gene expression of paired box 7 (Pax7), fibroblast growth factor-inducible 14 (Fn14), and tumor necrosis factor-α-receptor (TNFα-R), respectively. Similarly, perioperative markers of inflammation and myogenesis were associated with quadriceps strength at 8 weeks post-surgery relative to pre-surgery measures. Testosterone supplementation during energy deficit attenuated Fn14 gene expression and increased androgen receptor protein content at baseline, and upregulated expression of the myogenic regulatory factor MyoD and attenuated proteolytic gene expression after exercise and feeding.

In total, findings from Study 1 and Study 2 indicate integration of intramuscular inflammation, dietary protein intake, and testosterone-mediated intracellular signaling in the regulation of muscle mass during conditions of muscle wasting.

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Integrated Role of Intramuscular Inflammation, Dietary Protein, and Testosterone Supplementation in the Regulation of Skeletal Muscle Mass during Muscle Wasting Conditions

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List of Abbreviations

1-RM	1-repetition maximum
4E-BP1	4E binding protein 1
ACL	anterior cruciate ligament
AP	adequate protein
AR	androgen receptor
ARE	androgen response element
BCAA	branched chain amino acids
BMI	body mass index
BTB	bone-tendon-bone
BW	body weight
CSA	cross-sectional area
CT	computed tomography
DHT	dihydrotestosterone
DL	double leg
DXA	dual X-ray absorptiometry
E2	estradiol
EAA	essential amino acid
ECM	extracellular matrix
ED	energy deficit
EIEE	exercise-induced energy expenditure
eIF2B	eukaryotic initiation factor 2B
ERK1/2	extracellular signal-related kinase 1/2
FAK	focal adhesion kinase
FAP	fibro/adipogenic progenitor
Fn14	fibroblast growth factor-inducible 14
FOXO1a	forkhead box protein O1a
FSH	follicle-stimulating hormone
Gln	Glutamine
GSH	glutathione
GSK-3β	glycogen synthase kinase 3β

GSSG	oxidized glutathione
HHD	hand-held dynamometer
HMB	β-hydroxyl-β-methylbutyrate
HSP90	heat shock protein 90
IGF-1	insulin-like growth factor 1
IL-10	interleukin-10
IL-13	interleukin-13
IL-4	interleukin-4
IL-6	interleukin-6
IRS-1	insulin receptor substrate 1
IκBα	inhibitor κBα
JAK	Janus kinase
LH	luteinizing hormone
MAFbx	muscle atrophy F-box
MHC	myosin heavy chain
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MRF4	myogenic regulatory factor 4
MRI	magnetic resonance imaging
MSI	musculoskeletal injury
mTOR	mammalian target of rapamycin
MuIS	muscle inflammatory susceptibility
MuRF1	muscle ring finger 1
Myf5	myogenic factor 5
MyoD	myogenic differentiation 1
ND	nandrolone decanoate
NF-κB	nuclear factor-κB
NIK	NFκB-inducing kinase
NMES	Neuromuscular electrical stimulation
OKC	open kinetic chain
OP	optimal protein

p38 MAPK	p38 mitogen-activated protein kinase
p70S6K	p70 ribosomal protein S6 kinase
Pax7	paired box 7
PCNA +	proliferating cell nuclear antigen positive
PI3K	phosphoinositide 3-kinase
PLA	placebo
PVDF	polyvinylidene fluoride
RDA	recommended dietary allowance
REDD1	regulated in development and DNA Damage 1
REDD2	regulated in development and DNA Damage 2
ROM	range of motion
ROS	reactive oxygen species
rpS6	ribosomal protein S6
SHBG	sex-hormone binding globulin
SL	single leg
STAT3	signal transducer and activator of transcription 3
TCF-4	T-cell factor-4
TDEE	total daily energy expenditure
TEST	testosterone
THA	total hip arthroplasty
TKA	total knee arthroplasty
TNFα	tumor necrosis factor-α
TSC2	tuberous sclerosis complex 2
TT	total testosterone
TWEAK	TNF-like weak inducer of apoptosis
UC	usual care
UPS	ubiquitin proteasome system
WBAT	weight bearing as tolerated
WM	weight maintenance
ΔCt	Δ cycle threshold

CHAPTER 1

Introduction

Muscle atrophy occurs with reduced loading and neural activation of muscle after a musculoskeletal injury (i.e., disuse atrophy) (1, 2) and during energy deficit resulting from inadequate caloric intake (3). Muscle mass is regulated by rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) that fluctuate throughout the day in response feeding and fasting. A persistent negative net balance (i.e., MPS < MPS) during disuse or energy deficit due to decreased MPS, increased MPB, or both, underlies the associated loss of lean mass (3, 4). Muscle mass is also regulated by the repair and replacement of damaged fibers through myogenesis. The myogenic response involves activation of normally quiescent muscle stem cells (i.e., satellite cells) to form new multinucleated myotubes that fuse with existing fibers (5). Upstream regulation of myogenesis involves transient increases in local inflammatory cytokine expression that promote the activation, proliferation, and differentiation of satellite cells (6, 7). If intramuscular inflammation persists chronically, however, regenerative capacity is diminished and muscle atrophy ensues (8, 9). Interventions designed to modulate protein turnover and enhance myogenesis by attenuating excessive inflammatory signaling may preserve muscle mass under muscle wasting conditions.

Manipulating the amount and quality of dietary protein is one possible strategy to protect muscle during disuse after musculoskeletal injury. The proposed anti-inflammatory role of branched chain amino acids (BCAAs) (10), and the capacity of essential amino acid (EAA) supplementation to modulate satellite cell activity directly (11), suggests that manipulating dietary protein intake may enhance muscle regenerative capacity to promote recovery from injury. Incorporating high quality protein into the diet may also enhance the anabolic response to

protein ingestion to overcome disuse-induced deficits in postprandial MPS and protect muscle mass (12).

Exogenous testosterone administration has been shown to increase rates of fasted-state MPS (13, 14), enhance satellite cell activity (15), and attenuate inflammatory signaling (16). Given that the loss of lean body mass with severe energy deficit generally occurs with a concomitant suppression of the hypothalamic-pituitary-gonadal axis (17), testosterone supplementation may represent one strategy to modulate MPS, myogenesis, and inflammation to attenuate muscle losses under these conditions. However, the molecular impacts of exogenous testosterone administration during severe exercise- and diet-induced energy deficit remain unexplored.

This dissertation therefore aims to:

1.) Determine whether varied protein intakes (1.0 vs. 2.0 $g \cdot kg^{-1} \cdot d^{-1}$) for 2 weeks prior to anterior cruciate ligament (ACL) reconstruction surgery modulates perioperative markers of intramuscular inflammation and myogenesis;

2.) Associate muscle regeneration capacity at the time of ACL surgery (i.e., inflammatory and myogenic signaling) with postoperative recovery outcomes;

3.) Determine the effects of increasing postoperative dietary protein intake (~2.0 $g \cdot kg^{-1} \cdot d^{-1}$) on preserving muscle mass and function outcomes after ACL reconstruction; and

4.) Characterize intracellular signaling and gene expression mediating the hypertrophic effects of supplemental testosterone during a severe diet- and exercise-induced energy deficit.

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CHAPTER 2

Review of Literature

a. Inflammation and myogenesis in the recovery from skeletal muscle injury

Skeletal muscle damage and a corresponding loss of function occurs as a result of strains, contusions, lacerations, ischemia, burns, and even strenuous exercise. Musculoskeletal injuries requiring surgical interventions can also lead to extensive muscle damage. Damage to the muscle surrounding the hip or knee, for example, is inevitable during total hip or total knee arthroplasty (THA or TKA) procedures, respectively (1). Using a tourniquet to maintain a bloodless field during surgery also causes muscle trauma through the associated ischemia and reperfusion injury (2). Skeletal muscle has the intrinsic capacity to regenerate and repair itself following these types of injuries through myogenesis. This myogenic response involves the activation of muscle-resident stem cells (i.e., satellite cells) that ultimately regenerate and remodel the injured tissue.

In addition to muscle tissue damage itself, the loss of limb function and mobility with skeletal muscle injury induces a rapid loss of muscle mass (i.e., disuse atrophy) and function given the unloading and reduced neural activation of muscle. Rehabilitation begins gradually post-injury or post-operatively to attenuate these muscle losses and regain muscle mass, strength, and function in the injured limb. Restoring muscle mass is mediated by a positive muscle protein balance (i.e., muscle protein synthesis > muscle protein breakdown). Myogenesis also appears to be involved in restoring muscle mass as muscle fiber hypertrophy was compromised after 8 weeks of overloading in mouse muscle depleted of satellite cells (3). Myogenesis is therefore critical in post-injury or post-operative recovery to not only repair and regenerate

damaged muscle tissue, but to also add new nuclei to existing myofibers in support of muscle hypertrophy during rehabilitation (4).

Myogenesis is a complex and highly regulated process involving the activation of normally quiescent satellite cells to reenter the cell cycle and generate myoblasts in response to exercise, damage, or other stimuli (5). Myoblasts proliferate rapidly and differentiate by increasing expression of specific transcription factors (e.g. Pax7, Myf5, MyoD, myogenin, MRF4) (**Fig 2.1**). Differentiated myoblasts then fuse with each other or existing myofibers to regenerate and repair damaged tissue. Activation of fibro/adipogenic progenitor (FAP)-mediated remodeling of the connective tissue extracellular matrix (ECM) supports this myogenic response (6). Revascularization and reinnervation of damaged muscle tissue through angiogenesis and neuromuscular junction formation, respectively, is also necessary to restore muscle function post-injury (7).

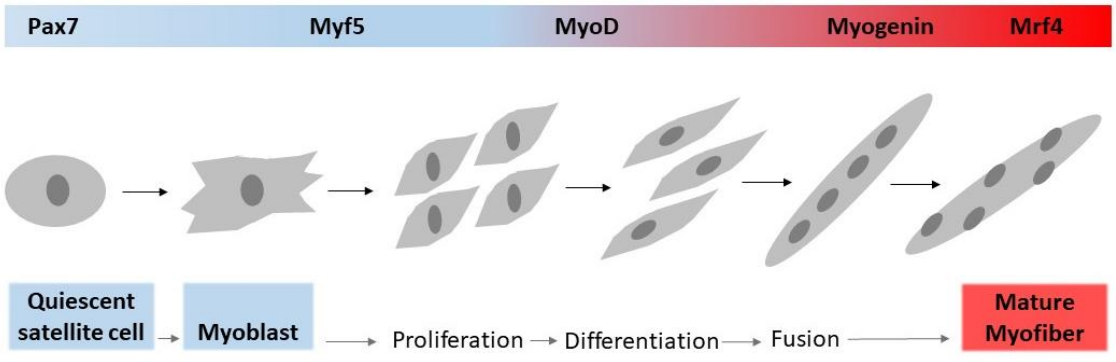

Figure 2.1. Myogenic regulatory factor expression mediates the progression of myogenesis

Regulation of the regenerative response is multifaceted and involves factors such as inflammatory cytokines, growth factors, immune cell infiltration, hypoxia and nutrient availability (8, 9). The inflammatory response, specifically, occurs rapidly in response to tissue

damage with the activation of the complement system. Neutrophils and macrophages infiltrate the injured area as a result to clear cellular debris. Inflammatory cytokines are then secreted by these immune cells and myofibers themselves, modifying the local microenvironment to promote satellite cell activation, proliferation, and differentiation (10).

The release of inflammatory cytokines following muscle damage is a finely regulated response. An early and continuous presence of several pro-inflammatory cytokines (e.g., TNFα, TWEAK) mediate early stages of muscle regeneration and repair (11, 12). This local elevation in pro-inflammatory cytokine expression persists for only a short time. A shift from pro- to anti-inflammatory signaling (e.g., IL-13, IL-10, IL-4) within days of muscle injury represses the local inflammatory response and supports later phases of myogenesis (13, 14). The regenerative response to muscle damage is therefore impaired with exceedingly high or chronic expression of pro-inflammatory cytokines. Disrupting the switch from pro- to anti-inflammatory signaling has been shown to increase ECM deposition and favor muscle degeneration and fibrogenesis (15). Persistent pro-inflammatory cytokine signaling may also promote muscle wasting by blunting muscle protein synthesis and triggering muscle protein breakdown (16, 17). While multiple cytokines are involved in regulating these processes, TWEAK, TNFα, IL-6 and their respective molecular pathways appear to be shared across conditions of both acute and chronic inflammation.

b. Divergent roles of inflammation in the regulation of muscle mass

Physiological and pathophysiological stress can trigger a muscle inflammatory response that promotes muscle growth or muscle loss, respectively. Transiently elevated muscle inflammation after routine exercise, for example, is a normal physiological response that regulates muscle regeneration and repair in healthy individuals. The cytokine TNF-like weak

inducer of apoptosis (TWEAK) and its receptor fibroblast growth factor-inducible 14 (Fn14) constitute one signaling pathway involved in this response. Levels of the TWEAK receptor Fn14 are generally low in healthy tissues and therefore the induction of Fn14 expression is tied to TWEAK/Fn14 pathway activity (18). Fn14 mRNA has been shown to increase in animals after skeletal muscle tissue injury, denervation of skeletal muscle, and in response to growth factors such as PDGF and VEGF (19). While literature examining the TWEAK-Fn14 axis in human skeletal muscle is limited, Raue and colleagues (20) were the first to observe increased intramuscular Fn14 gene expression in humans following exercise. Skeletal muscle Fn14 expression was induced after an acute resistance exercise bout in mixed muscle and in MHC IIa muscle fibers of young and old subjects (20). Its induction also positively correlated with skeletal muscle growth (cross sectional area) and functional improvements (1- repetition maximum) over 12 weeks of training (20). An Fn14 gene response in fast-twitch fibers has also been observed in highly trained college runners after tapered run training, a time when these muscle fibers increase in size (21). Collectively, these findings highlight a growth-related physiological role for the TWEAK-Fn14 axis in human skeletal muscle.

The pro-growth qualities of TWEAK/Fn14 signaling may explain the apparent benefit of heightened intramuscular inflammation during a period of muscle loss in a healthy population. Total body mass decreased by approximately 9% in a population of healthy males subjected to 21 days of high altitude exposure and a simultaneous energy deficit achieved through dietary restriction and multiple bouts of aerobic-type exercise (22). Approximately 43% of this loss in total body mass was attributed to a decrease in fat-free mass (22). Markers of muscle inflammation (e.g. TWEAK, Fn14, TNF-α, and TNF-α -R) and myogenic regulatory factors (e.g. Myogenin) were also upregulated during this time (22). Dichotomizing participants into HIGH

and LOW Fn14 expression revealed that those classified as HIGH lost significantly less fat-free mass and displayed increased myogenic regulatory factory gene expression versus those classified as LOW (22). These findings suggest that a heightened muscle inflammatory response to chronic high altitude exposure, underfeeding and exercise may benefit muscle maintenance under these conditions by promoting muscle regeneration.

In contrast, exceedingly high or chronic skeletal muscle inflammation may inhibit muscle regeneration and promote muscle wasting by blunting muscle protein synthesis and triggering muscle protein breakdown. This is evident in several pathophysiological conditions of muscle wasting. Burn injury, as an extreme example, leads to skeletal muscle breakdown throughout the body. A marked increase in vastus lateralis mRNA expression of TWEAK-, tumor necrosis factor-α (TNFα)- and interleukin (IL)-6-family cytokines and receptors 5-days post burn injury suggests changes in inflammatory signaling may contribute to the observed atrophy (23). Elevated expression of TWEAK and TNFα receptors has similarly been observed in paralyzed muscle of men with chronic spinal cord injury, a condition that also presents with muscle atrophy and impaired regenerative capacity (24).

i. Inflammation and myogenesis

The opposing roles of inflammation observed in humans correspond with the concentration- and time-dependent regulation of myogenesis by several inflammatory cytokines in cell and animal models (**Fig. 2.2**). TWEAK, for example, has emerged as a cytokine capable of modulating myogenesis. Initial studies demonstrated that high concentrations of exogenous TWEAK (≥ 100 ng/mL) in cultured myoblasts enhanced proliferation but inhibited subsequent differentiation and myotube formation (11, 25). The observed impairment in cell cycle exit and muscle specific gene expression in TWEAK-treated myoblasts corresponded with decreased

gene expression and protein levels of the myogenic regulatory factors MyoD and myogenin (11, 25). Activation of classical (canonical) nuclear factor-kB (NF-κB) signaling appears to mediate this response. NF-κB proteins are a family of structurally similar transcription factors (p65, RelB, c-Rel, p105/p50, and p100/p52) that form either homodimers or heterodimers. Classical signaling specifically involves the phosphorylation and degradation of an inhibitory protein (IκBα) and subsequent translocation of an activated p65/p50 heterodimer to the nucleus (26). Inhibiting p65/p50 activity reversed the inhibitory effect of soluble TWEAK (500 ng/mL) on differentiation and MyoD protein expression in cultured myoblasts (25), suggesting high concentrations of TWEAK limit normal myogenic progression through classical NF-κB activity.

High concentrations of TWEAK enhancing proliferation and inhibiting differentiation through classical NF-κB signaling would be critical during early phases of muscle regeneration to promote rapid proliferation and adequate expansion of the myoblast population, while simultaneously avoiding premature differentiation (18). In contrast, TWEAK present at low concentrations (10 ng/mL) in C2C12 myoblasts has been shown to promote later stages of myogenesis through the activation of alternative (noncanonical) NF-κB signaling (27). The alternative NF-κB signaling pathway involves the phosphorylation and partial proteasomal degradation of a p100 subunit to p52 to generate a p52/RelB heterodimer (26). Translocation of the p52/RelB heterodimer to the nucleus and subsequent transcriptional activity promotes myogenesis by stimulating myoblast fusion into myotubes. Exposing differentiating myoblasts to low concentrations of exogenous TWEAK (10 ng/mL) increased myoblast fusion and doubled myotube diameter when compared to untreated cells (27).

The cytokine TNFα also regulates myogenesis. TNFα concentrations rise substantially at the site of muscle injury due to its release from injured myofibers and infiltrating immune cells

(28). The early increase in TNFα expression functions as a chemoattractant signal that stimulates myogenic cell migration to the site of injury (29). The initially high concentrations of TNFα also play a role in promoting proliferation (12) and inhibiting myogenic differentiation (30). This occurs analogous to TWEAK through the activation of classical NF-κB signaling and downstream inhibition of MyoD mRNA and protein expression (30). While high concentrations of recombinant TNFα (≥ 0.5 ng/mL) inhibit the progression of myogenesis, low concentrations of TNFα (0.05 ng/mL) have been shown to enhance differentiation in cultured myoblasts (31). This response appears to occur through downstream activation of p38 MAPK. Levels of activated p38 MAPK and markers of differentiation were both diminished when TNFα was neutralized in C2C12 myoblast (31). p38 MAPK activation and muscle regeneration were also impaired following cardiotoxin-injury of soleus muscle in TNFα receptor double knockout mice ($p55^{-/-}p75^{-/-}$) (32).

The cytokine IL-6 is another major regulator of myogenesis. IL-6 binding to its receptor activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling cascade. IL-6-dependent activation of STAT3 specifically was required for satellite cell proliferation *in vitro* (33). IL-6 is also necessary for the complete differentiation of muscle cells. Primary muscle cells from IL-$6^{-/-}$ mice displayed a clear reduction in myotube formation indicative of decreased myoblast fusion (34). This occurred independent of downstream STAT3 activation, although the exact mediators of this effect are unknown. How exceedingly high levels of IL-6 affect myogenesis is unclear, however, chronic overexpression of IL-6 has been shown to induce muscle wasting (16).

In total, these findings specific to TWEAK, TNFα, and IL-6 indicate that high concentrations of these cytokines may inhibit normal myogenic progression. It is important to

note, however, that the physiological relevance of these *in vitro* studies remains unclear as they generally involve acute exposure to very high concentrations of a single cytokine. The involvement of multiple cytokines under physiological conditions and the varied concentrations of cytokines between plasma and muscle interstitium due to the presence of an endothelial barrier are possible confounding factors that must be considered. Regardless, these findings collectively suggest that a transient increase in intramuscular pro-inflammatory signaling after injury is required for muscle regeneration and repair, while an excessive or persistent inflammatory response can prevent myogenesis and limit recovery.

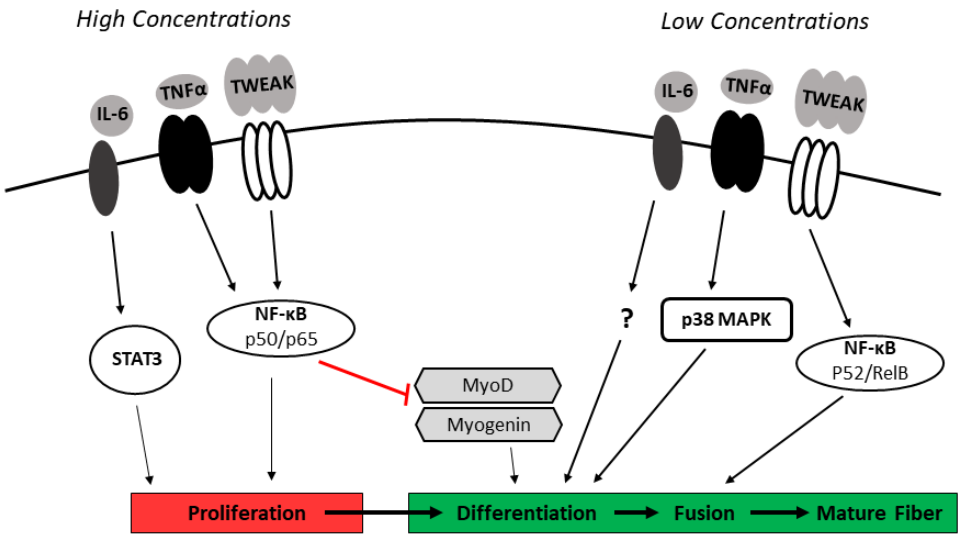

Figure 2.2. Inflammation and myogenesis

ii. Inflammation and muscle protein turnover

Exceedingly high or chronic expression of pro-inflammatory cytokines following muscle injury impairs recovery by promoting muscle wasting (**Fig. 2.3**). Differentiated C2C12 myotubes incubated with soluble TWEAK (10 ng/mL), for example, display reduced mass and a loss of total protein content (17). Mice subjected to chronic administration of soluble TWEAK similarly exhibit reduced fiber diameter in isolated muscle sections and decreased body weight

compared to control mice (17). This effect appears to involve multiple signaling pathways that promote protein catabolism and impair anabolic signaling. Activation of NF-κB, for example, is involved in the TWEAK-induced degradation of cultured myotubes (17). NF-κB signaling likely mediates this effect by up-regulating the ubiquitin proteasome system (UPS). The UPS is largely responsible for the degradation of myofibrillar proteins through the enzymatic activity of the muscle-specific ubiquitin ligases muscle atrophy F-box (MAFbx/Atrogin-1) and muscle ring finger 1 (MuRF1). NF-κB has been shown to regulate MuRF1 expression (35), which was increased along with MAFbx following TWEAK treatment of cultured myotubes (17).

Inhibition of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway may also contribute to the observed up-regulation of UPS activity in TWEAK-treated myotubes. Active Akt phosphorylates and inhibits the forkhead box O (FoxO) transcription factor, preventing its translocation to the nucleus and induction of MAFbx and MuRF1 expression (36). Exogenous TWEAK in cultured myotubes significantly decreased Akt and FOXO1a phosphorylation, suggesting increases in MAFbx and MuRF1 following TWEAK treatment are also due to Akt inhibition (17). TWEAK-mediated inhibition of the PI3K/Akt is also consequential to anabolic signaling. Akt facilitates translation initiation and muscle protein synthesis through the phosphorylation and activation of the mammalian target of rapamycin (mTOR) and subsequent activity of its downstream targets, p70S6K and 4E-BP1 (37). Active Akt also phosphorylates GSK-3β, reversing its inhibitory effect on the translation initiation factor eIF2B (38). Phosphorylation levels of GSK-3β, mTOR, and p70S6K were decreased following TWEAK treatment of cultured myotubes, indicating impaired anabolic signaling (17). An imbalance where catabolic signaling exceeds anabolic signaling can lead to muscle wasting.

Elevated levels of TNFα modulate muscle protein turnover and induce atrophy. A dose-dependent loss of total protein was noted in differentiated skeletal muscle myotubes following prolonged exposure to TNFα (1 – 6 ng/mL) (39). TNFα appears to mediate this effect by activating NF-κB transcriptional activity (16) and inhibiting Akt (42), triggering downstream up-regulation MAFbx and MuRF1 expression. The up-regulation of MAFbx in C2C12 myoblasts following TNFα exposure also required intact p38 MAPK, indicating the TNFα/p38 MAPK signaling axis enhances downstream UPS activity and related muscle catabolism (41). Increased TNFα concentrations have also been shown to limit muscle anabolism via the PI3K/Akt signaling pathway. Upstream activation of PI3K/Akt signaling involves the binding of factors such as insulin-like growth factor 1 (IGF-1) or insulin to their receptors and the subsequent phosphorylation and activation of insulin receptor substrate 1 (IRS-1). TNFα may exert its anti-anabolic effect by downregulating IGF-1 synthesis or by direct interaction with IRS-1. Addition of TNFα to cultured myotubes decreased IGF-1 mRNA expression by 50-80% (42). TNFα has also been shown to stimulate serine residue phosphorylation of IRS-1, preventing its recruitment to the insulin/IGF-1 receptor (43). In theory, this would ultimately suppress anabolic (i.e., mTOR, p70S6K) and promote catabolic (i.e., FOXO, MuRF1, MAFbx) signaling downstream of the IGF-1 receptor.

The effects of heightened IL-6 expression on muscle anabolic and catabolic signaling pathways *in vivo* remain elusive. Locally infusing soluble IL-6 in muscles of rats significantly decreased myofibrillar protein content compared to untreated contralateral muscle (16). Inhibiting STAT3 in C2C12 myoblasts reduced muscle atrophy downstream of IL-6, implicating the JAK/STAT3 pathway as a mediator of IL-6-induced muscle wasting (44). The mechanisms by which STAT3 promotes atrophy, however, are still unknown. IL-6-mediated muscle wasting

may also result from a downregulation of anabolic signaling pathways. An increase in IGF-1 mRNA and decrease in downstream phosphorylation of p70S6K has been observed in IL-6-infused muscles of rats (16). These findings suggest that IL-6 disrupts growth factor-related intracellular signaling, resulting in a compensatory increase in IGF-1 production (16).

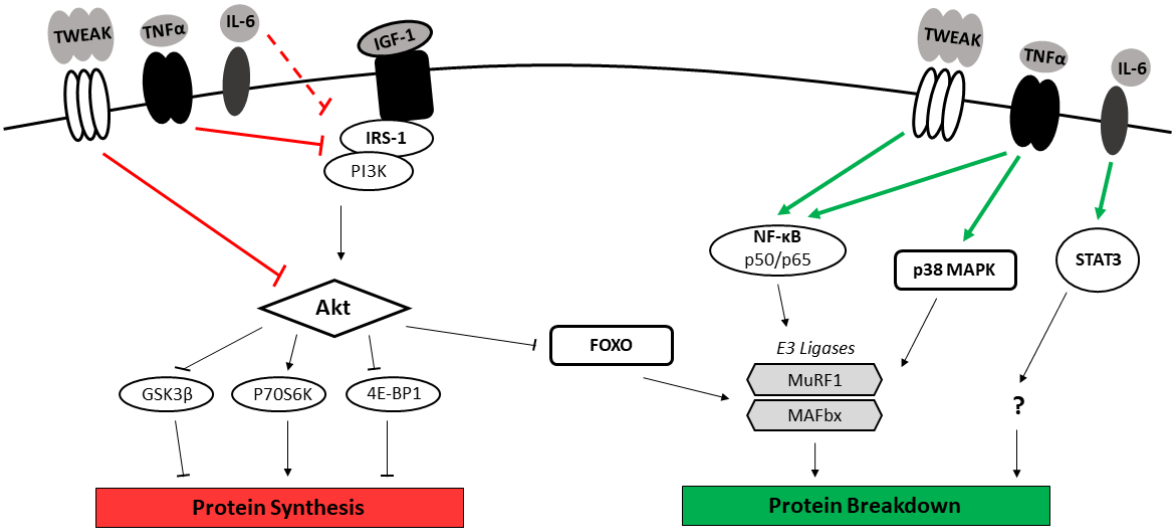

Figure 2.3. Inflammation and muscle protein turnover

c. Variability in muscle inflammation susceptibility

Heightened basal (pre-injury or pre-surgery) levels of intramuscular inflammation, an exaggerated inflammatory response to muscle damage, or both, would lead to exceedingly high levels of muscle inflammation post-injury that could impair myogenesis and promote atrophy. Interestingly, basal levels of inflammation and the sensitivity to inflammatory stimuli may not be uniform across all populations. Merritt and colleagues (45) observed differences in basal muscle pro-inflammatory signaling independent of circulating cytokines between middle-aged adults (40.4 ± 1.1 yr, AGE40), older adults (61.2 ± 0.6 yr, AGE61), and elderly (75.5 ± 0.7 yr, AGE76) individuals. IL-6, TNFα, and TWEAK expression were higher in AGE61 and AGE76 groups

relative to AGE40 at baseline and following modest muscle damage induced with a resistance exercise protocol (45). Myoblasts isolated from three young (28 ± 2 yr) versus three older (64 ± 2 yr) individuals also displayed greater inflammatory signaling in the absence of a pro-inflammatory stimulus, and had an exaggerated inflammatory response and reduced fusion capacity when treated with TNFα (45). This heightened basal pro-inflammatory signaling and hypersensitivity to inflammatory stimuli in individuals of advanced age was defined as "muscle inflammation susceptibility", or MuIS$^{(+)}$. This MuIS$^{(+)}$ phenotype, along with cell-intrinsic defects in satellite cell self-renewal observed with aging (46), impair myogenesis as well as promote muscle atrophy following muscle injury.

Follow-up work by Bamman et al. (47) described analyses identifying the MuIS$^{(+)}$ phenotype in certain adults across all ages, suggesting some individuals display elevated basal levels of muscle inflammation and an exaggerated or prolonged response to an inflammatory insult. This apparent interindividual variability in heightened inflammation susceptibility suggests some individuals may be more prone to an exceedingly high inflammatory response to muscle injury that would be detrimental to recovery. Bamman et al. (47) considered this hypothesis by evaluating MuIS status in patients undergoing total hip arthroplasty (THA) for end-stage osteoarthritis, since osteoarthritis-related damage in the hip leads to immune cell infiltration and heightened inflammatory cytokine expression in the synovial fluid, synovial membrane, cartilage, and the subchondral bone layer (48, 49). In theory, evaluating MuIS status would ascertain the susceptibility of muscle surrounding the hip to the local inflammatory burden of the osteoarthritic joint (47).

The MuIS$^{(+)}$ phenotype was identified in a population of THA patients based on the expression of the TWEAK receptor, fibroblast growth factor inducible 14 (Fn14), in muscle

surrounding the diseased hip (47). Levels of Fn14 are generally low in healthy tissues and therefore the induction of Fn14 expression in response to injury, stress, or exercise is tied to TWEAK/Fn14 pathway activity (18). Dichotomization into $MuIS^{(+)}$ and $MuIS^{(-)}$ individuals was done based on median Fn14 expression and revealed several key differences between groups. The $MuIS^{(+)}$ group (n=7) had a mean Fn14 gene expression that was five times higher than the $MuIS^{(-)}$ group (n=8) (47). Individuals designated as $MuIS^{(+)}$ also exhibited heightened expression of all inflammatory genes evaluated (e.g., TNFα, IL-6, TWEAK) compared to non-surgical controls, while only the up-regulation of the IL-6 receptor (IL-6R) was found in the $MuIS^{(-)}$ group (47). Significantly lower muscle protein synthesis (i.e., fractional synthetic rate) was observed in muscle surrounding the diseased hip in the $MuIS^{(+)}$ versus $MuIS^{(-)}$ groups (47). The heightened inflammatory signaling and changes in muscle protein synthesis in the $MuIS^{(+)}$ versus $MuIS^{(-)}$ group would be expected to reduce myogenic activity and induce muscle atrophy. These findings collectively suggest that dichotomization of individuals based on Fn14 expression reveals a phenotype that would be detrimental to recovery from THA, though long-term investigations evaluating the recovery potential of $MuIS^{(+)}$ versus $MuIS^{(-)}$ THA patients are currently lacking.

The possibility exists that these observations may not be unique to THA patients. Levinger and colleagues (50) observed heightened inflammatory cytokine expression in the vastus lateralis of patients undergoing total knee arthroplasty (TKA) for end-stage osteoarthritis. This observation is intriguing given the relative distance between the inflamed joint and surrounding musculature, and may be relevant to other orthopedic injuries and surgeries. Anterior cruciate ligament (ACL) injury and reconstruction, for example, has been associated with elevated inflammatory cytokine expression in synovial fluid of the injured knee (51). Based

on the findings in TKA patients, it is plausible that the local knee inflammation observed with ACL injury and reconstruction may similarly extend to the vastus lateralis. A heightened susceptibility to a locally inflamed joint (e.g., knee, hip, or shoulder) following musculoskeletal injury may contribute to an excessive inflammatory response to surgical treatment that limits regeneration of damaged tissue and promotes atrophy in the postoperative period. Whether this situation provides insight into a patient's rehabilitative potential is not known.

Potential problems with existing methods of determining MuIS status that currently limit its clinical application should be noted. Dichotomization based on Fn14 expression, for example, results in groups of individuals (i.e., above versus below the cut-off point) that are considered equal although their individual prognosis may vary considerably. Likewise, individuals close to, but on opposite sides of, the cut-off point are regarded as very different. Dichotomization based on the sample median also means that the exact value of the cut-off point could change considerably from sample to sample. Finally, the clinical application of this tool in its current form has methodical limitations. Collecting a muscle sample and evaluating gene expression is outside the scope of most clinical sites and laboratories. Nonetheless, potential benefits to determining MuIS status in orthopedic patient populations with specific regard for prognostic outcomes following surgical interventions warrants further investigations.

d. Dietary protein intake and myogenesis

The relationship between dietary protein intake and satellite cell-mediated muscle regeneration and remodeling is not fully understood. Some insight is provided by *in vitro* studies evaluating the effect of amino acid availability on the myogenic response. Adding branched chain amino acids (BCAAs) (52) or leucine alone (53) to culture medium of C2C12 myoblasts, for example, increased proliferation of these cells. Leucine treatment of primary rat satellite

cells similarly enhanced myotube formation and increased MyoD and myogenin expression (54), while the removal of leucine from culture media prevented differentiation of C2C12 myoblasts and primary satellite cells from mice (55). Leucine supplementation has also been shown to modulate satellite cell dynamics *in vivo*. Pereira and colleagues (56) provided leucine to young and old rats for 3 days before and 10 days after cryolesion-induced muscle damage. An increased number of proliferating cells (i.e., $MyoD^+$ satellite cells relative to the total number of $Pax7^+$ satellite cells) were found 3 days post-injury in both young and old rats receiving the leucine supplement compared to the controls (56). Type II fiber satellite cell proliferation was similarly increased in rats provided with the leucine metabolite β-Hydroxy-β-methylbutyrate (HMB) during 14 days of reloading after a period of disuse (57).

Current literature translating these findings to humans is limited, and is mainly focused on the relationship between protein or free amino acid supplementation and the exercise-induced satellite cell response in older adults. Farup et al. (58) found that consuming 28 g of whey protein after a bout of eccentric exercise increased satellite cell content in the vastus lateralis at 48 hours compared to the placebo group. The number of proliferating satellite cells 24 hours after a single bout of resistance exercise was also greater in elderly men who consumed 10 g of EAAs post-exercise versus those who did not (59). There may be a limited time course to the effect of protein supplementation on satellite cell activity. The acute satellite cell response to a single resistance exercise bout was not different in those consuming a multi-ingredient protein-based supplement (30 g whey protein, 2.5 g creatine, 400 mg Ca, 500 IU vitamin D, 1 g carbohydrate, and 0.75 g n-3 fatty acids) versus those consuming a carbohydrate-based placebo for 7 weeks prior to and 48 hours after the exercise bout (60). It's possible that exercise-induced satellite cell activity would have increased at earlier time points during the intervention, but

habituation to the supplement resulted in a similar satellite cell response to exercise between the groups at 7 weeks.

A protein-based intervention also appears to influence satellite cell dynamics when implemented before and after total knee arthroplasty (TKA). Patients that consumed 20 g of essential amino acids (EAAs) twice-daily beginning 7 days before their TKA procedure had increased satellite cell numbers at the time of surgery (61). This effect was short-lived even with continued supplementation, however, as the number of satellite cells in the EAA group declined and were similar to the placebo group at 7 and 14 days post-surgery (61). Habituation to the supplement is one possible explanation for these findings, although it is unclear if the surgery itself or the loss of muscle observed in the postoperative period also played a role. Differences in myogenic regulatory factor expression between groups also suggests that EAA supplementation after surgery may have altered the activation state of satellite cells without a corresponding effect on satellite cell number. Myogenin expression, for example, was increased at weeks 1 and 2 post-surgery in the EAA group and only at week 2 in the placebo group (61). Expression of this regulatory factor generally occurs in later phases of the myogenic response (62). This suggests a larger portion of the muscle precursor pool was in a later stage of myogenesis in the EAA versus the placebo group at week 1, possibly indicating an accelerated myogenic response (61). These findings are supported by MyoD mRNA expression which was only upregulated in the placebo group at 1 and 2 weeks postoperatively (61). MyoD is expressed before myogenin in activated and proliferating satellite cells (62), suggesting a larger portion of the muscle precursor pool in the placebo group may have been in earlier stages of myogenesis.

e. Dietary protein as a countermeasure to skeletal muscle inflammation

Immune cells localized to a site of skeletal muscle inflammation can generate reactive oxidative species (ROS) and cause oxidative stress (63). Excessive ROS production exacerbates skeletal muscle inflammation by activating redox-sensitive NF-κB signaling and downstream pro-inflammatory gene expression (64). The intracellular antioxidant Glutathione (GSH) protects against oxidative stress by reacting directly with ROS and producing oxidized GSH (GSSG). Maintaining adequate levels of GSH through recycling of GSSG and de novo synthesis is therefore necessary to protect against an excessive oxidative stress-related inflammatory response. De novo synthesis of GSH demands an adequate supply of glutamate (via glutamine), cysteine, and glycine that are linked in a 2-step process through action of glutamate cysteine ligase and glutathione synthase (65). The branched chain amino acids (BCAAs) leucine, isoleucine and valine are significant sources of nitrogen to glutamate and glutamine production via transamination (**Fig. 2.4**).

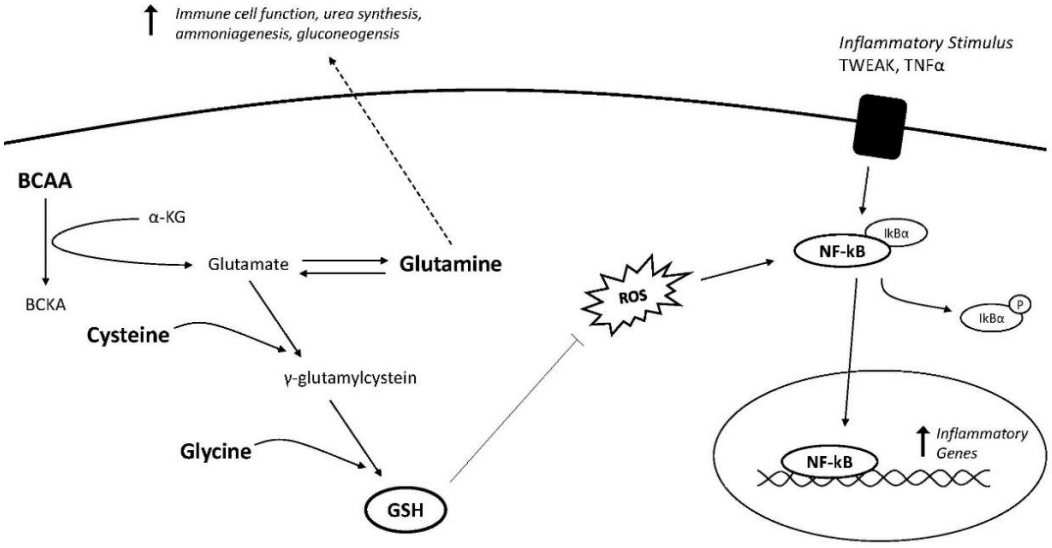

Figure 2.4 Protein intake modulates skeletal muscle inflammation

By providing an abundance of dietary BCAAs as well as glutamate, cysteine, and glycine, higher protein intakes may improve redox protection given the role of these amino acids

as GSH precursors. Whey protein, high in BCAAs and cysteine, has been shown to modulate GSH levels of muscle tissue *in vitro*. Treating C2C12 muscle cells with whey protein at various concentrations (.78 – 6.24 mg protein/mL) increased GSH levels by 25.7% and 138% at the low and high concentrations respectively (66). The antioxidant potential of whey protein is also evident in cystic fibrosis patients whose lung inflammation and associated oxidative stress increases the demand for GSH. Consuming 10 g of whey versus 10 g of casein twice daily for 3 months increased circulating lymphocyte GSH concentrations by 46.6% (67).

Increasing the supply of exogenous BCAAs through higher protein intakes may also augment glutamine availability and have immunomodulatory effects. Transamination of BCAAs with α-ketoglutarate in skeletal muscle generates glutamate that can be used to produce glutamine with the incorporation of a free NH_3. Immune system cells require the amino acid glutamine to function optimally. Glutamine addition to cell culture medium increased phagocytic activity and enhanced superoxide production in neutrophils and monocytes isolated postoperatively from gastrointestinal surgery patients (68). Rate of lymphocyte proliferation was similarly accelerated *in vitro* in the presence of increasing extracellular glutamine concentrations (69). An abundance of exogenous BCAAs serving as nitrogen donors for skeletal muscle glutamine production has therefore been hypothesized to elicit a more efficient and accelerated immune response (70). In support of this concept, Pereira and colleagues (71) observed an attenuated inflammatory response and enhanced muscle recovery after muscle injury in rats consuming the BCAA leucine. Supplemental leucine 3 days before and 10 days after cryolesion-induced muscle damage in young rats attenuated the number of macrophages and area of inflammatory cell infiltration at the site of injury (71). A similar investigation with older rats demonstrated that older rats not consuming the leucine supplement had an area of inflammatory

cell infiltration approximately 44% larger than their younger counterparts 10 days after cryolesion-induced muscle damage (56). Supplemental leucine negated this age-related difference in area of inflammation, suggesting leucine supplementation has some capacity to attenuate an excessive inflammatory response to muscle injury.

Current literature translating these findings to humans is limited. Muyskens and colleagues (61) provide some of the only insight through their work involving supplementation of TKA patients with either 20 g of EAAs or a placebo twice daily for 7 days before and 6 weeks after surgery. Similar to the findings in rats, EAA supplementation after surgery attenuated M1 macrophage area in vastus lateralis tissue compared to the placebo group (61). Inflammatory cytokine expression was also different between groups. TNFα mRNA expression in the EAA group was elevated 1 week postoperatively and declined by week 2 (61). In contrast, TNFα was only elevated at week 2 in the placebo group (61). These findings collectively suggest that the immune response differed between groups and appears attenuated or accelerated in the EAA group.

f. Disuse atrophy as a consequence of musculoskeletal injury

Acute or repetitive stressors that overload, overstretch, or deform tissues of the musculoskeletal system (i.e. muscle, tendon, bone, ligaments) can result in MSI and a corresponding loss of function. Common injuries include muscle strains or contusions, muscle or tendon tears, ligament sprains, joint dislocations, and bone fractures. Surgical interventions required to treat some MSIs can also cause further injury. Total hip arthroplasty (THA) procedures, for example, involve unavoidable trauma to muscle surrounding the hip (1). Tourniquet use during surgery also causes muscle damage through the associated ischemia and reperfusion injury (2).

Injury-related trauma disrupts normal function of the involved muscle, connective tissue or joint. Inflammation, pain, and swelling of the affected area causes an immediate decline in range of motion, neuromuscular signaling, and strength that can persist well after injury. Knee extensor strength in anterior cruciate ligament (ACL) reconstruction patients, for example, was 66.9% lower one month postoperatively in the injured versus non-injured limb (72). These changes in strength and functional capacity post-injury can be explained in part by underlying deficits in neuromuscular signaling. Musculoskeletal damage and the associated inflammatory response alters sensory stimuli, receptor activity, and signal transduction from the injured area to the central nervous system. This is evident following knee joint damage, as swelling, inflammation, joint laxity, and damage to articular sensory receptors alters afferent signaling from the joint that limits maximal voluntary contraction of the quadriceps muscle (73).

The loss of neuromuscular signaling and the resulting decline in function following MSI constitutes a natural protective response that limits additional structural damage. Protective measures such as prescribed inactivity, immobilization with a cast or brace, and unloading via crutches or bed rest may also be implemented to restrict motion and prevent further injury. While the loss of neuromuscular signaling and interventions to limit movement aid the healing process after MSI, muscle atrophy and weakness occurs rapidly under these conditions given the disuse of muscle. This is evident in experimental models of disuse that have shown a 3.5 and 8.4% decline in quadriceps cross-sectional area (CSA) in healthy young individuals after only five and 14 days of one-legged knee immobilization, respectively (74). Single-leg 1- repetition maximum (1-RM) strength also declined by 9% after 5 days of immobilization and 22.9% after 14 days (74).

The disproportionately greater loss of strength compared to muscle mass during disuse indicates there is also a loss of muscle quality (i.e., muscle strength relative to muscle size). This may be explained in part by disuse-induced decreases in fascicle length and pennation angle that limit the capacity of muscle fibers to generate force (75). Diminished muscle quality may also result from changes at the single fiber level. A decrease in single fiber specific force (i.e., maximal Ca^{2+}-activated force relative to CSA) of isolated vastus lateralis fibers was observed following four (76) and 14 (77) days of unilateral leg immobilization. A loss of actin or myosin protein content (78, 79), changes in Ca^{2+} sensitivity (76), or other modifications of the contractile apparatus that alter active cross-bridge formation may explain the loss of single fiber and whole muscle quality with disuse.

g. Mechanisms of muscle disuse atrophy

Skeletal muscle mass is regulated by rates of MPS and MPB that fluctuate throughout the day in response to feeding and fasting. Hyperaminoacidemia in the postprandial period following protein ingestion transiently stimulates MPS (80), while a simultaneous hyperinsulinema inhibits MPB (81, 82). This effect is short-lived, however, as breakdown predominates in the postabsorptive period between meals. Collective rates of synthesis and breakdown and the resulting net balance (MPS minus MPB) control changes in muscle mass. A negative net balance (MPS < MPB) maintained over time and resulting from diminished rates of MPS, increased MPB, or both, would account for the muscle atrophy observed with disuse.

i. Muscle protein synthesis and anabolic signaling in disuse atrophy

Altered rates of MPS have consistently been observed during periods of disuse in young populations. Gibson and colleagues (83) were the first to demonstrate a 25% reduction in fasted state MPS after ~37 days of full leg casting for tibial fracture. Subsequent studies revealed this

decline in postabsorptive MPS occurs rapidly and persists with disuse of longer duration. Postabsorptive rates of MPS were 40% lower in immobilized versus control limbs after only 5 days of a full leg cast (84), and were similarly cut in half following 14 days of bed rest (85). The muscle protein synthetic response to protein intake is also blunted with periods of disuse. This "anabolic resistance" to protein ingestion occurs early, as rates of MPS following ingestion of 25 grams of whey protein were ~53% lower in immobilized versus control limbs after only 5 days of a full leg cast (84). Fourteen days of immobilization similarly blunted rates of MPS with both low and high dose amino acid infusions (86).

The time course and magnitude of disuse-related impairments in postprandial MPS are different between young and old populations. While the synthetic response to feeding was maintained after 5 days of bed rest in young individuals (22 ± 1 yr), rates of MPS were unresponsive to essential amino acid (EAA) ingestion in older adults (66 ± 1 yr) under the same conditions (87). Quantifying anabolic resistance by evaluating the fate of dietary-derived protein (i.e., incorporation into body proteins or irreversible hydroxylation) also revealed greater anabolic resistance in old versus young individuals with two weeks of bed rest (88). Age-related differences in postprandial synthetic responses have also been observed under normal conditions. Older adults displayed diminished responsiveness of MPS to graded intakes of EAAs (89) and required greater amounts of protein to maximally stimulate MPS after resistance exercise versus their younger counterparts (90). Inherent differences in the synthetic response to protein intake between old and young individuals (i.e., anabolic resistance with aging) may therefore explain the greater anabolic resistance with disuse in older adults, and suggests an age-related susceptibility to the negative effects of disuse on postprandial anabolism.

Cellular mechanisms underlying these changes in MPS with disuse are not fully understood. Protein synthesis is primarily regulated by activation of the mammalian target of rapamycin (mTOR) and phosphorylation of several downstream substrates (i.e., 4E-BP1, p70S6K, rpS6) that promote mRNA translation initiation. However, activation of this pathway in the postabsorptive state remains unchanged with disuse of short (84) and longer duration (86, 91), indicating the disuse-induced attenuation of postabsorptive MPS occurs independent of changes in mTOR-stimulated translation initiation. Whether disuse alters the regulation of elongation (i.e., translation efficiency) or ribosomal biogenesis (i.e., translation capacity) downstream of mTOR has not been well characterized in humans and should be a focus of future work. Regulation of protein synthesis can also occur independent of the mTOR pathway through phosphorylation of glycogen synthase kinase (GSK)-3β and downstream activation of the translation initiation factor eukaryotic initiation factor 2B (eIF2B). Immobilization for 48 hours decreased GSK-3β phosphorylation by 21% (92), suggesting alterations in GSK-3β signaling may be linked to the attenuation of postabsorptive MPS with disuse. It is unclear, however, if GSK-3β phosphorylation is decreased with disuse of longer duration or if downstream eIF2B phosphorylation is similarly altered.

Although mTOR signaling appears unrelated to the attenuation of postabsorptive MPS, blunted activation of this pathway may explain disuse-related impairments in postprandial MPS. Bed rest of five (87) or seven (93) days in older adults blunted the phosphorylation of p70 ribosomal protein S6 kinase (p70S6K) and ribosomal protein S6 (rpS6) with EAA ingestion. EAA-stimulated mTOR signaling was similarly attenuated after 5 days of bed rest in a young population, however, this occurred without changes in MPS or lean mass (87). It was speculated this decrease in intracellular anabolic signaling may have preceded alterations in protein

synthesis and lean mass that would have been observed in bed rest of longer duration (87). A change in postprandial mTOR signaling was also observed in young individuals after 5 days of unilateral leg immobilization. Phosphorylation of mTOR and 4E binding protein 1 (4E-BP1) was decreased relative to fasted values 4 hours post-protein ingestion in immobilized versus control limbs (84). Given that peak stimulation of mTOR signaling is generally observed 1-2 hours after feeding, the authors concluded the depressed levels of phosphorylated mTOR and 4E-BP1 at 4 hours indicated reduced transduction of the anabolic signal through this pathway (84).

While upstream regulators of disuse-induced anabolic resistance are poorly defined, focal adhesion kinase (FAK) is a mechanosensitive protein that may connect the loss of muscle contraction during disuse with impairments in postprandial mTOR signaling. Mechanical stimuli are sensed by membrane-associated integrin receptors and transmitted via phosphorylation of FAK and other downstream kinases (94). Basal levels of FAK phosphorylation appear sensitive to loading as FAK phosphorylation was increased with chronic resistance exercise (95) and decreased with disuse of short (96) and longer duration (86, 91). This load-dependent activation of FAK may affect mTOR signaling. Phosphorylated FAK promotes mTOR activity *in vitro* by phosphorylating and inhibiting tuberous sclerosis complex 2 (TSC2), a negative regulator of mTOR (97, 98). A decline in FAK phosphorylation and possible increase in active TSC2 with disuse may therefore explain the disuse-induced attenuation of postprandial mTOR activity and rates of MPS. Further *in vitro* and *in vivo* investigations are needed, however, to firmly establish this relationship.

The attenuation of mTOR pathway activity underlying anabolic resistance with disuse may also result from cellular stress. The protein regulated in development and DNA Damage (REDD) 1/2 has been implicated in the cellular response to various stressors (i.e., hypoxia,

immobilization) and acts to repress mTOR signaling (99). An upregulation of REDD2 mRNA expression with disuse was first observed following immobilization in rodents (100), and also occurred with 5 days of bed rest in young and old individuals (87). These findings suggest that increased expression of REDD2 may be involved in the attenuation of postprandial mTOR signaling. REDD1 mRNA expression was also upregulated with bed rest (87). This only occurred in young individuals, however, indicating the effect of REDD1 on mTOR signaling during disuse may be age-dependent.

ii. Muscle protein breakdown and catabolic signaling in disuse atrophy

While deficits in MPS are a well-recognized consequence of disuse, less is known about the role of protein breakdown in disuse-induced muscle atrophy. Rates of MPB are seldom reported given the technical challenges associated with directly measuring protein degradation *in vivo* in humans. To our knowledge only two studies have measured rates of MPB during disuse conditions and reported no changes with 14 (85) and 21 (101) days of bed rest in young individuals. These findings complement calculations suggesting alterations in MPS alone sufficiently account for the loss of muscle mass during disuse and have led to the hypothesis that disuse atrophy occurs without appreciable changes in MPB (85, 102). This contrasts indirect measures of MPB (i.e., mRNA expression of proteolytic proteins) that appear to change with short-term unloading and may indicate some role of MPB during disuse.

Breakdown of muscle protein is regulated by the autophagy-lysosomal system, calcium-dependent calpains, caspase enzymes, and the ubiquitin proteasome system (UPS). The UPS, in particular, is largely responsible for the degradation of myofibrillar proteins through enzymatic activity of the muscle-specific ubiquitin ligases muscle ring fiber 1 (MuRF1) and muscle atrophy F-box (MAFbx)/atrogin-1 (103). Transcript levels of MAFbx and MuRF1 are increased under

several conditions of muscle atrophy (104). Immobilization of 2-14 days, for example, elevated mRNA expression of MAFbx and/or MuRF1 in young individuals (84, 91, 105-107). In contrast, transcript levels of these proteins remained unchanged or decreased compared to baseline in other studies of disuse lasting 14 - 24 days (78, 91, 107). The time-dependent expression of these static markers of proteolysis suggest MPB increases early with the onset of disuse and returns to baseline with inactivity of longer duration as hypothesized by Wall and colleagues (108). Changes in proteolytic gene expression do not always translate to changes in protein content (109) or measured rates of MPB (82), however, indicating future work must use dynamic measures of proteolysis (i.e., stable isotope methodology) to confirm the proposed early increase of MPB rates with disuse.

Disuse may also alter postprandial rates of MPB. Insulin released with protein feeding modulates glucose metabolism and suppresses MPB (81, 82). Sensitivity to this insulin stimulus appears to decline with disuse, however, as impairments in whole-body and muscle glucose uptake in response to insulin were observed with bed rest (110) and immobilization (111), respectively. Whether disuse leads to insulin resistance of protein metabolism is less clear. Work by Richter et al. (111) using a two-step euglycemic hyperinsulinemic clamp procedure showed that the normal hyperinsulinemia-induced attenuation of MPB may be impaired with 7 days of immobilization. Arterial tyrosine concentrations were measured to assess net protein breakdown as tyrosine is not synthesized or catabolized in muscle. The greater tyrosine release with insulin infusion in immobilized versus control limbs suggests MPB was less sensitive to inhibition by insulin under disuse conditions (111). Whether these findings extend to protein feeding during disuse is unknown, however, given current methodical limitations associated with directly measuring MPB following protein ingestion.

h. Protein as a countermeasure to muscle disuse atrophy

Attenuating muscle losses and preserving muscle function with disuse may require interventions to overcome the observed decline in MPS (postabsorptive and postprandial) and possible early increase in MPB. Muscle protein turnover is modulated primarily by dietary protein in the absence of muscle contraction. Optimizing the anabolic response to protein intake may therefore be one way to increase MPS and overcome anabolic resistance to preserve muscle mass with disuse. Twenty grams of high-quality protein have been shown to maximally stimulate MPS under normal physiological conditions in young individuals (112), while greater amounts (~35 g) were needed to overcome the anabolic resistance observed with aging and achieve maximal rates of MPS in an elderly population (113). Larger quantities of protein may similarly be necessary to overcome disuse-induced anabolic resistance. Other strategies to overcome anabolic resistance include manipulating the EAA and/or leucine content of the diet. Considerable work has shown that the EAA component of protein is responsible for stimulating MPS (114, 115), while leucine in particular has been identified as a 'trigger' for the synthetic response (116). Several studies have therefore manipulated the protein, EAA, or leucine content of the diet in an effort to attenuate muscle atrophy during a period of experimental disuse (i.e., bed rest or immobilization in healthy populations) with mixed results.

Protein or amino acid supplementation has been shown to protect lean mass in several studies of disuse. Early work by Paddon-Jones et al. (117) found that supplementing a controlled diet with 16.5 g of EAAs plus 30 g of sucrose three times a day maintained leg lean mass and partially preserved strength in young adults subjected to 28 days of bed rest. Leucine supplementation (4.4 g per meal) similarly attenuated declines in knee extensor peak torque, endurance, and muscle quality (peak torque/kg leg lean mass) with 14 days of bed rest in middle-

aged adults (118). Whole-body lean mass was only preserved during the first week, however, suggesting a limited time course for the protective effect of leucine supplementation on lean mass during disuse (118). Supplementation with the leucine metabolite β-hydroxyl-β-methylbutyrate (HMB) during 10 days of bed rest also preserved whole body lean mass (119). Protein intake was only at the current recommended dietary allowance (RDA) of 0.8 g/kg/d in the control group, however, which may be inadequate in older populations (120). The benefit of the intervention may therefore reflect adequate protein intake rather than a protective effect of HMB itself (121).

Additional literature has reported a muscle-preserving effect of protein-based interventions without a corresponding change in muscle function. Consuming ~66.8 g of a proprietary amino acid-containing formulation on top of a diet providing 1.0 g/kg/d, for example, attenuated muscle losses with 7 days of immobilization, while declines in knee extensor strength remained unaffected by the intervention (122). A trend for the partial protection of leg lean mass ($p=0.08$) with no change in strength outcomes was also observed when high-quality whey protein was incorporated into the diet of healthy older men and women during 7 days of bed rest (123). In contrast, EAA supplementation has also been shown to preserve muscle function during disuse with no effect on lean mass losses. Older adults consuming 15 g of EAA three times daily during 10 days of bed rest lost similar amounts of total and leg lean mass compared to the control group, while muscle function outcomes (e.g., floor transfer time) were only preserved in those consuming the EAA supplement (124).

Although protein or amino acid supplementation appears to have some benefit during disuse, it is not always protective under these conditions. Daily supplementation of 20 g of whey protein during 14 days of unilateral leg immobilization, for example, did not attenuate disuse

atrophy or protect isometric knee extension strength, single leg jump height, or peak power production during an incremental cycling test (125). Dirks et al. (126) similarly observed no benefit of twice daily consumption of a leucine-enriched whey protein supplement on protecting lean mass or single-leg 1-RM strength during 5 days of a full leg cast in older adults. Adding 2.5 g of leucine to every meal during 7 days of immobilization in young adults also had no effect on preserving muscle mass or function (127) despite the previously reported benefit during 7 days of bed rest (118). Dividing up and consuming an additional 3.6 g leucine, 1.8 g valine, and 1.8 g isoleucine over 3 meals during 60 days of bed rest was also ineffective, and actually lead to a greater loss of thigh muscle volume compared to the control group (128).

The heterogeneity of these studies makes it challenging to precisely understand why muscle mass and/or muscle function is protected in some cases but not others. Differences in model and duration of disuse, subject age, and outcomes measured may all contribute to the inconsistent findings. Protein-based interventions during disuse of varying durations (5 – 60 days) may not be comparable given the proposed difference in mechanisms underlying short- versus long-term disuse (108). Results from different age groups (i.e., young, middle-aged, and elderly adults) should also be compared cautiously given the previously discussed susceptibility of older adults to anabolic resistance during disuse that may influence the effectiveness of particular interventions. Differences in methods used to measure muscle mass and muscle function during disuse may also contribute to variable findings. Results obtained from isokinetic dynamometry and 1-RM testing, for example, were not equivalent in assessing the efficacy of a resistance training intervention (129) suggesting studies using these different methods to evaluate the loss of strength with disuse may be difficult to compare.

The wide variety of protein and free amino acid supplementation protocols tested during disuse may also contribute to inconsistent findings. There is currently a poor understanding of what would constitute an optimal protein-based intervention. While one objective of these countermeasures is to overcome disuse-induced anabolic resistance, no study to date has determined if this can be done with greater amounts of protein, EAAs or leucine, or if there is a threshold for maximally stimulating postprandial MPS with disuse (130). Whether the different protein or free amino acid interventions tested in current literature had the capacity to overcome disuse-induced anabolic resistance is therefore unclear. Some insight comes from Wall et al. (84) who showed that postprandial rates of MPS following ingestion of 25 g of whey protein (10.9 g EAA, 2.7 g Leucine) were cut in half with 5 days of a full leg cast. These findings suggest the ~21 g of leucine-enriched whey protein (10.6 g EAA, 2.8 g leucine) consumed immediately after breakfast and before sleep during 5 days of immobilization in a separate study (126), may not have been sufficient to overcome anabolic resistance in the absence of additional protein consumed at these time points. This may explain the similar loss of quadriceps CSA and 1-RM strength observed in the protein supplement and control group.

Further understanding of mechanisms underlying the loss of muscle mass and function with disuse may also clarify inconsistent findings and explain why some interventions maintained muscle mass and/or function and why others were ineffective. As discussed previously, it is unknown if disuse leads to an early increase in MPB or if postprandial rates of MPB are greater with disuse. Whether an abundance of exogenous amino acids from protein or amino acid supplements would spare the breakdown of body proteins in the fed and/or fasted state or if interventions should target this is or not is unclear. Future work should also establish how some protein-based interventions preserve muscle function during disuse in the absence of

corresponding changes in muscle mass. It is possible that stimulating protein turnover in these studies (118, 123) facilitated the removal and replacement of aged or damaged proteins in what has been described as 'nonhypertrophic' remodeling (131), resulting in better muscle quality and improved function of contractile units that would explain the preserved function. This remains to be determined, however.

i. Protein to support rehabilitation following musculoskeletal injury

While protein-based interventions appear to have some capacity to protect skeletal muscle mass and function with disuse, the most effective countermeasure is simply muscle contraction in the form of electrical stimulation or resistance exercise. Muscle contraction is a potent stimulus for MPS, even in disused muscle. Gibson et al. (132) were the first to show that daily percutaneous electrical stimulation of quadriceps muscle during 6 weeks of immobilization for tibial fracture was sufficient to maintain rates of postabsorptive MPS in the immobilized limb. Exercising knee extensors to volitional muscle failure every other day using a horizontal leg-training device similarly attenuated the decline of fasted-state MPS observed with 14 days of bed rest (133). Exercise also sensitizes skeletal muscle to the anabolic effect of protein ingestion (134), suggesting disuse-induced anabolic resistance may similarly be attenuated with some form of muscle contraction. The effect of exercise on rates of MPS translates to the preservation of muscle mass during disuse. Neuromuscular electrical stimulation (NMES) for 40 minutes twice a day preserved quadriceps CSA during 5 days of one-legged knee immobilization (105). Thigh CSA and isometric knee extensor strength were similarly preserved during 14 days of immobilization by low-volume, high-intensity resistance exercise (i.e., 80% 1-RM) (135).

Protecting muscle during disuse conditions using exercise or electrical stimulation is not a new idea in clinical settings. This is reflected in standard rehabilitation protocols that seek to

introduce exercise and activation of muscle as early as possible after MSI. Given the physical constraints associated with injuries themselves, resistance exercise at workloads shown to increase MPS (133) or preserve muscle mass and function during disuse (135) are generally not possible. Early rehabilitation instead focuses on mobilization and activation of muscle through body weight or light-load resistance exercise often performed to fatigue. Surrogates for muscle contraction (i.e., NMES) are also generally incorporated into standard rehabilitation. Unloading and disuse of muscle following MSI is therefore accompanied by periodic muscle activation during rehabilitation. This must considered when translating and implementing findings from protein-based interventions in experimental disuse conditions to disuse following MSI.

Whether light-load resistance exercise or electrical stimulation of muscle during rehabilitation has a stimulatory effect on rates of MPS or improves sensitivity of muscle to protein ingestion is not completely understood. Some insight comes from Wall and colleagues (136) who showed that one hour of high-frequency, high-intensity NMES in elderly men increased postabsorptive MPS by ~27% compared to the non-stimulated leg. The pre-sleep application of NMES plus ingestion of 40 g of protein after 1 day of bed rest also increased overnight MPS, suggesting NMES can potentiate the anabolic response to protein intake in disuse conditions (137). The anabolic potential of light load resistance exercise normally and with disuse is less clear. A single bout of low intensity resistance exercise (i.e., 16% 1-RM) comparable to what would be performed during rehabilitation was unable to increase rates of myofibrillar protein synthesis in the fed state or potentiate the synthetic response to feeding (138). Twelve weeks of resistance training at this same intensity led to a small (~3%) but significant hypertrophy of muscle, however, indicating an anabolic potential for this type of exercise (139). Follow-up analyses revealed that prior light load resistance exercise prolonged

the elevation of myofibrillar protein synthesis during 10 hours of hyperaminoacidemia, suggesting there was an increased sensitivity of muscle to protein feeding that allowed more amino acids to be stored as contractile proteins (140). These findings indicate that electrical stimulation of muscle and light-load resistance exercise in early rehabilitation combined with protein-centered diet interventions would have a synergistic effect at increasing rates of MPS, overcoming anabolic resistance, and protecting muscle mass and function from disuse after injury.

While research translating this concept to practice by implementing protein-based diet interventions during early rehabilitation from MSI remains limited, available literature suggests some benefit to free amino acid supplementation in the pre- and post-operative period following orthopedic surgery. Consuming 20 g of EAAs twice a day for 7 days before and 6 weeks after total knee arthroplasty (TKA), for example, attenuated the loss of quadriceps muscle volume when compared to the placebo group at 2 weeks (-3.4 ± 3.1 % vs. -14.3 ± 3.6 %) and 6 weeks (-6.2 ± 2.2 % vs. -18.4 ± 2.3%) postoperatively (141). EAA supplementation also accelerated the return of functional mobility (i.e., timed up-and-go, stair-climb up, and stair-climb down tests) at 6 weeks compared to the placebo group (141). These findings must be interpreted with caution, however, given the decrease in total amount of dietary protein consumed in the placebo group during the intervention. While protein intake was close to the current RDA of 0.8 g/kg/d at baseline and 6 weeks, it fell to 0.63 ± 0.09 g/kg/d in the placebo group at 2 weeks post-surgery (141). This may have exacerbated the loss of muscle volume and function observed in these individuals. A follow-up study of the same intervention also observed a decrease in protein intake from baseline to week 2, however the reduction was similar in both group (142). Quadriceps muscle atrophy was also attenuated at 6 weeks in this study in the EAA versus

placebo group (-8.5±2.5% vs. -13.4±1.9%), while differences between groups in functional measures or strength were no longer observed (142). Ferrando et al. (143) also evaluated the utility of EAA supplementation after orthopedic surgery by randomizing total hip arthroplasty (THA) patients to receive usual care (UC) or to consume 15 g of EAAs three times a day for 8 weeks after surgery. Quadriceps maximal voluntary contraction was 35% greater than preoperative values at 8 weeks post-surgery in the EAA group, while strength did not improve in those receiving UC (143). Supplementation with 2.4 g of HMB, 14 g of L-arginine, and 14 g of L-glutamine (HMB/Arg/Gln) twice daily for 5 days before and 28 days after TKA also has been shown to benefit strength outcomes. Maximal quadriceps strength declined from baseline at 2 weeks post-surgery in the control group, while the HMB/Arg/Gln intervention preserved strength at this time point (144).

j. Testosterone-mediated regulation of muscle mass

Exogenous testosterone administration has consistently been shown to induce skeletal muscle hypertrophy. Replacement doses of testosterone enanthate (100 mg/wk) led to greater arm and leg muscle cross-sectional area (CSA) in healthy, hypogonadal men (145), and increased muscle volume in HIV-infected men with weight loss and low testosterone levels (146). Muscle hypertrophy has also been observed in eugonadal men receiving supraphysiological doses of testosterone. Weekly administration of 125, 300 or 600 mg of testosterone enanthate along with monthly injections of a long-acting gonadotropin-releasing hormone (GnRH) agonist to suppress endogenous testosterone secretion, dose-dependently increased fat-free mass in healthy young men after 20 weeks by 3.4, 5.2, and 7.9 kg, respectively (147). Although the hypertrophic effect of exogenous testosterone administration is well-established, the underlying mechanisms are less clear.

Muscle mass is regulated by rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) that fluctuate throughout the day in response to anabolic (i.e., feeding) and catabolic (i.e., fasting) stimuli. A persistent positive net balance (MPS > MPB) resulting from an increase in MPS, decrease in MPB, or both, would lead to lean mass accretion over time. Muscle mass is also regulated through activity of normally quiescent adult muscle stem cells (i.e., satellite cells) that repair or remodel muscle tissue when activated by forming new multinucleated myotubes that fuse with existing fibers (4, 131). Current literature suggests testosterone exerts its hypertrophic effect by acting on MPS, MPB, or satellite cell activity.

i. Testosterone and myogenesis

Exogenous testosterone administration has been shown to modulate satellite cell dynamics in animal models and in humans. Testosterone treatment before the onset of puberty in male and female rats, for example, transiently increased satellite cell content of androgen-responsive muscle groups and increased the number of myonuclei (148). Sinha-Hikim et al. (149) similarly observed a dose-dependent increase in satellite cell number following weekly administration of 300 or 600 mg of testosterone enanthate in men given a GnRH agonist, along with greater muscle CSA and myonuclear number. The increased number of satellite cells and their subsequent fusion with muscle fibers likely gave rise to the greater myonuclear content observed in both studies given that muscle fiber nuclei are postmitotic. These findings collectively indicate a direct regulation of satellite cells by testosterone that may underlie its hypertrophic effect in muscle.

While it appears testosterone increases the number of satellite cells and their fusion with existing fibers, the mechanisms underlying this effect are not fully understood. Activated satellite cells reenter the cell cycle and generate myoblasts that proliferate and differentiate

rapidly in response to the sequential expression of specific transcription factors (i.e., Pax7, Myf5, MyoD, myogenin, and MRF4). A portion of the activated satellite cells also return back to quiescence to replenish the stem cell pool. Testosterone administration in humans has been shown to increase the number of proliferating cell nuclear antigen positive (PCNA +) satellite cells and the expression of myogenin in muscle, indicating testosterone promotes cell cycle entry and later stages of myogenesis (150). These findings are supported by *in vitro* models showing testosterone enhanced proliferation (151, 152) and differentiation (151, 153) of cultured myoblasts. Additional *in vitro* studies have reported no change in proliferation (153, 154) or reduced differentiation (154) with testosterone administration, indicating future work is needed to define a clear mechanism of action. Testosterone may also contribute to the formation of new myotubes via action on uncommitted, pluripotent mesenchymal stem cells that are capable of differentiating into muscle, fat cartilage, and bone cells. Incubating mouse mesenchymal C3H 10T1/2 cells with testosterone or dihydrotestosterone (DHT) dose-dependently increased the mRNA and protein levels of MyoD and myosin heavy chain II (MHC), indicating testosterone can recruit mesenchymal pluripotent cells into the myogenic lineage (155).

The androgen receptor (AR) appears to mediate some of the effects of exogenous testosterone administration on satellite cells activity and the myogenic response. Expression of AR has been shown to increase in muscle following androgen treatment in rats (156) and humans (157). While AR is found in several cell types, satellite cells are the predominant site of AR expression in human skeletal muscle (158), suggesting the increase in AR following testosterone treatment may be linked to changes in satellite cell activity. AR exerts an effect by binding androgens that cross the cell membrane. The resulting complex translocates to the nucleus and alters transcriptional activity by interacting with androgen response elements (ARE) of target

genes. Lee et al. (153) have reported a relationship between androgen-AR signaling and the upregulation of myogenin expression in C2C12 myoblasts. Androgens also appear to regulate myogenic differentiation of mesenchymal multipotent cells by inducing an interaction between AR and β-catenin to activate T-cell factor-4 (TCF-4) target genes (159).

Testosterone may also promote satellite cell proliferation and differentiation through AR-independent mechanisms. Proliferation and differentiation were enhanced in skeletal muscle myoblast L6 cell lines (L6 cells) lacking the classical AR following the addition of exogenous testosterone or a form of testosterone that wouldn't cross the cell membrane (151). This effect was determined to be dependent on G-protein coupled receptors at the plasma membrane (151). This is in line with work from Estrada et al (160) showing testosterone induced a rapid change in intracellular Ca^{2+} levels through G-protein coupled receptor activity in rat myotubes, and suggests testosterone may promote satellite cell proliferation and differentiation through AR-independent signaling pathways.

Androgen action on satellite cell dynamics may also occur though the modulation of upstream inflammatory signaling. While a transient increase in local pro-inflammatory cytokine expression mediates the repair and regeneration of damaged myofibers through myogenesis, exceedingly high or chronic inflammation can inhibit the myogenic response and induce muscle atrophy. *In vitro* studies have shown that the production of the inflammatory cytokines TNFα, IL-1β, and IL-6 in human macrophages (161) and monocytes (162) is attenuated with the addition of testosterone to culture media. Androgen treatment in prostate cancer cells has also been shown to suppress activity of NFκB (163), a regulator of pro-inflammatory cytokine expression. This anti-inflammatory effect of testosterone has also been observed in human muscle. The upstream activator of NFκB signaling, NFκB-inducing kinase (NIK), was

decreased in skeletal muscle of elderly men following 7 days of testosterone treatment (164). Whether the modulation of inflammation by testosterone influences satellite cell activity is unclear.

ii. Testosterone and muscle protein synthesis

Exogenous testosterone administration has been shown to alter dynamic rates of protein synthesis under fasted, but not fed, conditions. Protein synthesis was increased and breakdown was unchanged, for example, five days after intramuscular injection of 200 mg of testosterone enanthate in healthy, young men (165). Increased rates of protein synthesis following an overnight fast were also observed in older men (~ 67 y) given testosterone injections for four weeks (166). It appears that exogenous testosterone administration does not change synthetic rates following amino acid feeding (167, 168). Protein synthesis was also unchanged when the supplementation protocol raised levels of androgens from a lower physiological/hypogonadal range to the normal physiological range (157, 167). These findings suggest that the effects of exogenous testosterone on rates of protein synthesis are related to testosterone concentration prior to measurement (i.e., hypogonadal versus supraphysiological) and metabolic state of an individual (i.e., fed versus fasted) (169).

Molecular mechanisms underlying the effect of androgens on protein synthesis are not fully understood. An increase in protein synthesis following an anabolic stimulus involves signaling through mTOR and the phosphorylation of several downstream targets (i.e., 4E-BP1, p70S6K, rpS6) that promote mRNA translation initiation. Increased signaling through this pathway and modulation of upstream effectors such as insulin-like growth factor-1 (IGF-1)/phosphatidylinositol 3-Kinase (PI3K)/Akt and extracellular signal-related kinase 1/2 (ERK1/2) have been observed with testosterone administration *in vitro*. Testosterone-induced

myotube hypertrophy in primary rat myoblasts was accompanied by activation of ERK1/2 and Akt, as well as downstream S6K1 phosphorylation (170). Inhibiting PI3K/Akt or mTOR negated the increase in CSA observed at 12 hours (170). Inhibiting AR also attenuated myotube CSA, suggesting AR as well as Akt/mTOR signaling are essential in coordinating the hypertrophic response to supplemental testosterone (170). In line with these findings, Wu et al. (171) reported that testosterone-induced increases in protein synthesis were blocked by the mTOR inhibitor rapamycin. Androgen withdrawal in rats through castration or nandrolone decanoate (ND) treatment also decreased myofibrillar protein synthesis through Akt/mTOR signaling (172).

It is unclear if the relationship between testosterone and mTOR signaling observed *in vitro* translates to humans. Although cell and animal studies have identified a link between testosterone and mTOR pathway signaling, this work may not accurately reflect the fasted conditions under which testosterone-induced increases in protein synthesis have been observed in humans (169). Rossetti et al. (169) have suggested that mechanistic studies must employ periods of fasting in animal models and serum or nutrient deprivation prior to harvesting cultured cells to more accurately understand molecular signaling underlying the androgen-mediated increase in protein synthesis observed under fasted metabolic conditions. Whether mTOR signaling regulates rates of synthesis during basal, non-stimulated conditions is not fully understood. Work from Dickenson et al. has shown that treating humans with the mTOR inhibitor rapamycin only altered rates of muscle protein synthesis in the fed (173) but not the fasted state (174). This suggests other signaling pathways may be involved in regulating rates of protein synthesis under fasted conditions.

iii. Testosterone and muscle protein breakdown

Androgen-mediated changes in MPB have been observed in some, but not all, studies of exogenous testosterone administration in humans. Testosterone enanthate injection in older men (~67 y) whose pretreatment testosterone levels were in the lower physiological/hypogonadal range, for example, decreased rates of MPB (157, 167). In contrast, MPB was unchanged five days after a single testosterone or oxandrolone injection in young males (165, 168). Differences in age as well as pretreatment and posttreatment androgen levels (i.e., hypogonadal to physiological vs. physiological to supraphysiological) may explain these contrasting outcomes (169). Whether testosterone influences MPB after feeding is unknown given current methodical limitations associated with directly measuring MPB following protein ingestion.

The ubiquitin proteasome system (UPS) is largely responsible for the degradation of myofibrillar proteins through enzymatic activity of the muscle-specific ubiquitin ligases MAFbx and MuRF1. These markers of MPB are altered in some, but not all, studies of exogenous testosterone supplementation. Transcript levels of MAFbx and MuRF1 were not altered with administration of supraphysiological concentrations of testosterone (100 nM) in primary rat myotubes (170) or in soleus muscle of androgen-treated mice (175). In contrast, MAFbx and MuRF1 expression increased in androgen-sensitive rat skeletal muscle with castration, and was fully repressed with acute testosterone administration (176). Work by Zhao et al. (177) has also shown that testosterone represses MAFbx expression through AR-dependent signaling in C2C12 cells. Discrepancies between studies may be related to choice of model or the metabolic state of animals (i.e., length of fasting before sacrifice) (169).

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CHAPTER 3

Perioperative markers of intramuscular inflammation and myogenesis are associated with muscle recovery outcomes following Anterior Cruciate Ligament reconstruction: potential role of dietary protein intake

Introduction

Quadriceps muscle atrophy and weakness are well-recognized clinical outcomes of anterior cruciate ligament (ACL) reconstruction that can persist after surgery despite aggressive physical rehabilitation efforts (1, 2). Failing to reverse these deficits in muscle mass and strength can negatively impact activities of daily living, physical performance, and quality of life. The etiology of postoperative muscle atrophy and weakness is complex. Deficits in neuromuscular signaling as well as protective measures implemented to avoid further injury (i.e., immobilization, inactivity) induce a rapid loss of muscle mass and function due to the unloading and reduced neural activation of muscle (3, 4). The involvement of intramuscular inflammatory signaling and satellite cell content (i.e., regenerative potential) in the muscle atrophy and loss of function observed in the postoperative period is less clear.

Repair or replacement of damaged muscle fibers via myogenesis contributes to the maintenance of muscle mass and function (5-7). This nonhypertrophic remodeling involves activation of muscle resident stem cells (i.e., satellite cells) and the coordinated expression of several regulatory factors (i.e., Pax7, Myf5, MyoD, myogenin, and MRF4) that initiate their subsequent proliferation and differentiation. Exceedingly high or chronic inflammation can inhibit this myogenic response and induce muscle atrophy. High concentrations of tumor necrosis factor-α (TNFα) and TNF-like weak inducer of apoptosis (TWEAK) in cultured myoblasts, for example, enhanced proliferation (8, 9), but inhibited myogenic differentiation (10,

11) and led to a loss of total protein content (12, 13). Infusion of soluble interleukin 6 (IL-6) into rat hindlimb muscle similarly decreased myofibrillar protein content compared to untreated contralateral muscle (14). Bamman and colleagues (15) hypothesized that increased muscle inflammation, altered satellite cell dynamics, or both, would exacerbate the disuse-induced loss of muscle mass and function and limit muscle regenerative capacity during postoperative recovery.

Intramuscular inflammatory signaling and the abundance or activity of satellite cells may be influenced by dietary protein intake. The number of proliferating satellite cells 24 hours after a single bout of resistance exercise was greater in individuals who consumed 10 g of essential amino acids (EAAs) post-exercise versus those who did not (16). Twice daily EAA supplementation for 1 week prior to total knee arthroplasty (TKA) also increased satellite cell content of vastus lateralis muscle on the day of surgery versus a placebo (17). Findings from cell and animal models suggest that an abundance of branched chain amino acids (BCAAs) in dietary protein may improve redox protection and attenuate oxidative stress-related inflammatory signaling given the role of these amino acids as precursors for the intracellular antioxidant Glutathione (GSH) (18-20). While research translating these effects to humans is limited, the possibility that manipulating protein intake preoperatively would promote satellite cell activity and attenuate intramuscular inflammation to optimize postoperative muscle recovery potential exists.

The primary objective of this study was to determine whether a high quality protein-based diet intervention providing ~2.0 g protein·kg^{-1}·d^{-1} for two weeks prior to ACL reconstruction would modulate skeletal muscle inflammation and increase myogenic regulatory factor expression at the time of surgery compared to diet providing 1.0 g protein·kg^{-1}·d^{-1}. A

secondary analysis of a subset of subjects was also performed to assess the relationship between inflammatory and myogenic signaling on the day of surgery and postoperative recovery outcomes (i.e., quadriceps strength and thigh circumference). We hypothesized that: 1) higher protein intakes before surgery would improve recovery potential by attenuating inflammation and promoting satellite cell activation and 2) that exceedingly high intramuscular inflammation and lower myogenic regulatory factor expression at the time of surgery would be associated with greater declines in muscle mass and strength in the early postoperative period.

Methods

Subjects

This study was approved by the Institutional Review Boards at Hartford Hospital (Hartford, CT) and the University of Connecticut (Storrs, CT). All subjects were informed of the purpose of the study, the experimental protocol, and all potential risks before giving written informed consent to participate. Five men aged 19 - 39 who were scheduled to undergo their first ACL reconstruction were recruited to participate. Subjects were identified by two participating surgeons and subsequently screened by study personnel for all eligibility requirements. Exclusion criteria included body mass indexes (BMIs) greater than 30, metabolic or cardiovascular abnormalities, food allergies, and gastrointestinal disorders (i.e., lactose intolerance). Individuals reporting use of nutritional/herbal supplements, anabolic steroids, and tobacco products were also excluded from participation.

Experimental design

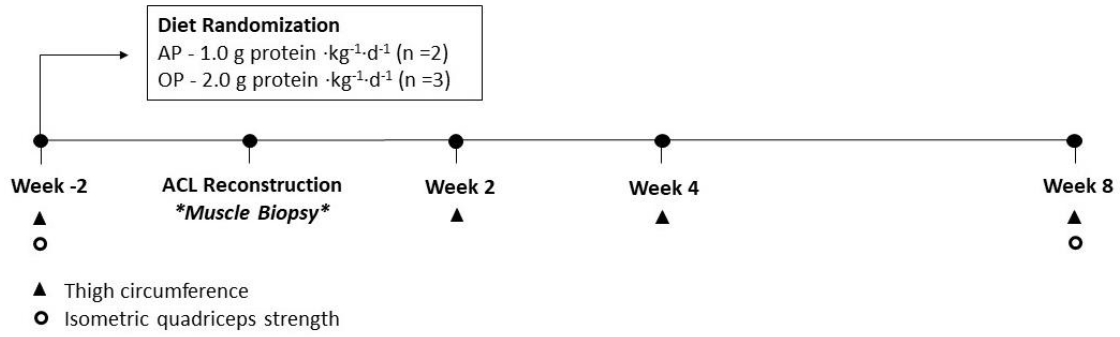

Figure 3.1. Experimental Design

This study was a two-arm, parallel trial design with subjects randomly assigned (1:1 randomization) to an adequate protein (AP) or optimal protein (OP) group providing 1.0 or 2.0 g protein·kg^{-1}·d^{-1}, respectively (**Fig. 3.1**). The diet intervention began 2 weeks prior to surgery and continued for 16 weeks postoperatively during which subjects participated in twice-weekly physical therapy. It should be noted that only data through the first 8 weeks of rehabilitation are presented for the purpose of this study to highlight the effects of protein and inflammation on early recovery outcomes. Thigh circumference was measured at baseline before surgery and again at 2, 4 and 8 weeks postoperatively. Maximal quadriceps isometric strength (i.e., peak force) was measured at baseline before surgery and again at 8 weeks. All recovery outcomes were measured by the same physical therapist assistant who was well-trained in all study procedures. A muscle biopsy of the vastus lateralis was collected at the time of surgery for skeletal muscle protein and gene expression analyses.

Diet interventions

Subjects followed a eucaloric (weight maintenance) diet providing 1.0 versus 2.0 g protein·kg^{-1}·d^{-1} (AP vs. OP), 30% of energy intake from fat, and remaining calories from carbohydrates starting 2 weeks before surgery and continuing for 16 weeks postoperatively. One subject in the OP group began the diet 1 week prior to surgery due to scheduling conflicts.

Individual energy requirements were established relative to estimated resting energy expenditure (Harris-Benedict equation), level of physical activity, and estimated energy intake reported at baseline. Subjects were given 1-week cycle menus individualized based on energy requirements and information obtained about their routine food consumption. Five servings (3 oz) of beef per week were provided as a high-quality protein to individuals in the OP group. Post-rehabilitation supplements were integrated into the overall diet design with one serving of IsoPrime Beef™ or an isocaloric serving of Powerade® provided to the OP and AP groups, respectively, for consumption after physical therapy sessions. Analyses determining the amino acid and macronutrient composition of the beef-based supplement were performed by Covance Laboratories Inc. (Madison, WI) and summarized in **Table 3.1**. Subjects consumed 4 servings of their designated supplement at home during the 2-week diet intervention before surgery to maintain consistency with the twice weekly supplement post-rehabilitation in the postoperative period. A 7-day food record beginning 1 week prior to surgery was collected to evaluate dietary intake during the pre-surgery diet intervention. Food records were analyzed using Nutritionist Pro™ software (Axxya Systems, Woodinville, WA) to estimate total energy and protein intake, as well as branched chain amino acid content of the diet.

Table 3.1. Isoprime Beef™ supplement composition

Calories (kcal)	100	**Amino Acids** (mg)			
Total Fat (g)	0	Aspartic Acid	1490	Leucine	957
Total Carbohydrate (g)	1	Threonine	525	Tyrosine	271
Protein (g)	25	Serine	753	Phenylalanine	589
		Glutamic Acid	2620	Lysine	976
		Proline	2770	Histidine	259
		Glycine	4800	Arginine	1810
		Alanine	1960	Cystine	14
		Valine	650	Methionine	248
		Isoleucine	434	Tryptophan	51

ACL reconstruction surgery

Subjects were admitted on the morning of surgery in a fasted state. They were initially anesthetized with intravenous propofol and maintained with inhalation anesthetic (sevoflurate) during surgery. Subjects also received regional anesthesia preoperatively consisting of an adductor canal and geniculate nerve block using 0.25% Marcaine with 1:200,000 epinephrine. A pneumatic tourniquet was applied 'as-high-as-possible' on the thigh of the injured limb during sterile preparation and draping. The lower extremity was exsanguinated and the tourniquet was inflated to a pressure of 250 mm Hg immediately prior to incision. The tourniquet was deflated following the graft harvest and muscle biopsy in three subjects (~16 minutes) and not until the end of surgery in two (~70 minutes). All ACLs were arthroscopically repaired with a bone-tendon-bone (BTB) autograft. One subject had a ruptured ACL with a concomitant meniscal root tear that also required surgical repair. While this did not influence the analysis of muscle tissue collected on the day of surgery, a meniscal repair necessitates a more prolonged period of restricted weight bearing and range of motion to protect the healing tissue. This individual was therefore excluded from analyses involving postoperative thigh circumference and strength given the altered rehabilitation and recovery after surgery. The remaining four subjects were non-weight bearing until their first postoperative visit with the physician (~ 7 days). This consisted of immobilizing the knee with a leg brace locked between 0° and 10° of flexion and using crutches. Subjects were then cleared to progress with weight bearing as tolerated (WBAT) and to begin twice weekly physical therapy.

Postoperative rehabilitation protocol

All rehabilitation was done by the same physical therapy assistant and following the Orthopedic Associates of Hartford return-to-sport after ACL reconstruction protocol (**Appendix**

I) and phase-specific exercises (**Appendix II**). Subjects were considered noncompliant with the rehabilitation protocol if they missed more than 4 physical therapy sessions total or two sessions in a row during the full 16-week diet intervention.

Muscle biopsy

Approximately 100 – 200 mg of vastus lateralis muscle tissue was collected during surgery with Rongeur forceps through the incision used for the ACL reconstruction. This occurred within 5 minutes of tourniquet inflation at the beginning of surgery. Collected muscle was carefully dissected to remove all visible fat and connective tissue and placed in a vial that was snap frozen in liquid nitrogen. Samples were stored at -80°C until later analyses.

mRNA expression

TRIzol reagent (ThermoFisher, Waltham, MA) was used to isolate total RNA from ~20 mg of muscle to determine expression of several genes linked to skeletal muscle inflammation and myogenesis. Quantity and quality of isolated RNA was assessed using a NanoDrop ND-2000 spectrophotometer (NanoDrop, Wilmington, DE). Equal amounts of total RNA (500 µg) were reverse-transcribed into cDNA (High-Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA) using a T100^{TM} Thermal Cycler (Bio-Rad, Hercules, CA). Samples were run in 10 µL reactions and in duplicate using TaqMan® fast advanced master mix in a Step One Plus Real-Time PCR system (Applied Biosystems). mRNA expression levels of IL6 receptor (IL-6R, TNFα receptor (TNFα-R), TWEAK, fibroblast growth factor-inducible 14 (Fn14), paired box 7 (Pax7), MyoD, Myogenin, myogenic factor 5 (Myf5), and myogenic regulatory factor 4 (MRF4) were determined using commercially available TaqMan® probes (Applied Biosystems). Data were normalized to the geometric mean of β-actin and β2 microglobulin to obtain Δ cycle threshold (ΔCt) values that were then compared between groups and correlated to recovery

outcomes. Data were analyzed this way as opposed to using the ΔΔCt method (21) given the lack of a healthy control population and the greater variance that would be introduced using the AP group (n=2) as a control.

Intracellular signaling

The relative abundance and phosphorylation status of proteins involved in intracellular inflammatory and myogenic signaling, as well as muscle protein synthetic and proteolytic pathways were determined using Western blot analysis. Approximately 20 mg of skeletal muscle was homogenized in ice-cold lysis buffer with protease and phosphatase inhibitors. Supernatant (lysate) was collected following the centrifugation of homogenates (15 min at 10,000 g at 4°C) to estimate protein concentrations (660 nm Protein Assay; Thermo Scientific, Rockford, IL). Muscle lysates were solubilized in Laemmli buffer, and equal amounts of total protein (20 µg) were separated by SDS-PAGE using precast Tris-HCl gels (Bio-Rad). Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and incubated overnight at 4°C with commercially available primary antibodies specific to MyoD and Myogenin (Santa Cruz Biotechnology, Santa Cruz, CA), total rpS6 (Abcam, Cambridge, MA), p-rpS6$^{Ser240/244}$, total FOXO1, p-FOXO1^{Thr24}, total p38 MAPK, p-p38 MAPK$^{Thr180/Tyr182}$, total NFκB p65, p-NFκB p65^{Ser468}, total IκBα, and p-IκBα$^{Ser32/36}$ (Cell Signaling Technology, Danvers, MA). Labeling was subsequently performed using anti-rabbit IgG conjugate with horseradish peroxidase (Cell Signaling Technology). Blots were quantified using ChemiDoc XRS (Bio-Rad) and Image Lab software (Bio-Rad) following the application of chemiluminescence reagent (Pierce, Rockford, IL). Heat-shock protein 90 (HSP90) was used to confirm equal loading of protein per well. Total protein normalized to HSP90 and the ratio of phosphorylation-to-total protein were compared between groups.

Isometric quadriceps strength

A belt-stabilized handheld dynamometer (HHD) (Lafayette Instruments, Lafayette, IN) was used to assess isometric quadriceps strength in the injured leg at baseline before surgery and at 8 weeks postoperatively. While isokinetic dynamometry is considered the gold standard for strength assessment, this method of handheld dynamometry has been shown to be reliable and valid in comparison (22-24). Using a procedure described by Hansen et al. (22), subjects were seated with legs positioned at 90° and thighs stabilized to the table using a standard gait belt. A second gait belt was positioned on the distal third of the tibia and used to stabilize the HHD behind the leg of the treatment table. Five maximal isometric contractions were performed with at least 1 minute rest between each contraction. Subjects were verbally encouraged to push the dynamometer with increasing force for 5 seconds during each trial. The first two trials were used as practice, while peak force during the final 3 contractions was averaged for each subject. Peak quadriceps isometric strength at week 8 was calculated as a percentage of baseline values.

Thigh circumference

Measurements of thigh circumference were used to estimate changes in muscle mass after ACL reconstruction. While muscle volume and cross sectional area (CSA) are generally assessed via magnetic resonance imaging (MRI), computerized tomography (CT) scans, or dual X-ray absorptiometry (DXA) in research settings, the cost and medical expertise required to use this equipment limits access to these measures in clinical practice (25). Alternatively, anthropometric measures (i.e., thigh circumference) are routinely used to roughly estimate changes in muscle mass. Thigh circumference was therefore evaluated during postoperative rehabilitation and included as a recovery outcome given that it was part of standard patient care. Measurements were made 15 cm from the superior pole of the patella in the injured limb. The

differences between pre-surgery baseline values and measurements at week 2, 4, and 8 were calculated.

Statistical analysis

Unpaired t-tests were used to examine differences in anthropometric measures, gene expression, and protein abundance between patients in the AP (n=2) and OP (n=3) groups. Effect sizes were also calculated given the small sample size. A post hoc power analysis using the program *G*Power* (26) was subsequently conducted to estimate the sample size needed for group differences to reach statistical significance at the 0.05 level with power set at 0.80. Pearson correlation coefficients were also calculated in a subset of subjects (n=4) to evaluate the relationship between intramuscular inflammation and myogenic regulatory factor expression at the time of surgery and postoperative muscle recovery outcomes (i.e., thigh circumference and quadriceps strength). As discussed, the patient with an ACL rupture and concomitant meniscal root tear was excluded from these analyses given the altered rehabilitation and recovery after surgery. The α level of significance for all statistical tests was two-tailed and set at $P < 0.05$, while trends were noted when $0.05 < P < 0.10$. Data were analyzed using IBM SPSS statistics for Windows Version 26 (IBM Corp. Armonk, NY, USA) and are presented as mean ± standard deviation (SD) within the text and figures.

Results

Subjects

Subject baseline characteristics are shown in **Table 3.2**. The 5 male subjects were 29 ± 9 years old on average and had a mean BMI of 24.9 ± 2.0 which did not differ between groups ($P > 0.05$).

Table 3.2 Baseline characteristics for study participants in AP and OP groups

	AP (n=2)	OP (n=3)
Age (yr)	29 ± 14	29 ± 7
Height (m)	1.85 ± 0.0	1.78 ± 0.1
Weight (kg)	89 ± 7	77 ± 13
Body mass index (kg/m^2)	25.9 ± 2.1	24.2 ± 2.1
Tourniquet Time (min)	49 ± 42	29 ± 27

Values mean ± SD. Baseline characteristics were not different between groups

Dietary Intake

Pre-surgery dietary intake is summarized in **Table 3.3**. Average protein intake derived from dietary records was greater in the OP versus AP group (1.9 ± 0.2 g/kg/d OP vs 1.2 ± 0.0 g/kg/d AP, p = 0.02). The average caloric intake of 37.4 ± 4.7 kcal/kg/d in the OP group also tended to be higher than the 27.3 ± 3.7 kcal/kg/d in AP individuals (p=0.08). Absolute and milligram per kg amounts of the BCAAs (i.e., leucine, isoleucine, and valine) were not different between groups (**Table 3.4**).

Table 3.3 Preoperative absolute, relative, and percent of energy and macronutrient intakes for AP and OP groups

	AP	OP	p-value
Absolute			
Energy (kcal/d)	2415 ± 133	2853 ± 382	0.23
Carbohydrate (g/d)	316 ± 46	313 ± 88	0.97
Protein (g/d)	104 ± 9	143 ± 19	0.08
Fat (g/d)	87 ± 1	110 ± 42	0.51
Relative			
Energy (kcal/kg/d)	27.3 ± 3.7	37.4 ± 4.7	0.08
Carbohydrate (g/kg/d)	3.6 ± 0.8	4.3 ± 1.7	0.64
Protein (g/kg/d)	1.2 ± 0.0	1.9 ± 0.2	0.02
Fat (g/kg/d)	1.0 ± 0.1	1.4 ± 0.3	0.14
Percent Total Energy			
Carbohydrate (%)	52 ± 5	45 ± 15	0.55
Protein (%)	17 ± 2	20 ± 0.2	0.12
Fat (%)	33 ± 2	34 ± 9	0.82

Values mean ± SD

Table 3.4 Preoperative BCAA intake for AP and OP

	AP	OP	p-value
Absolute (g/d)			
Leucine	6.13 ± 1.37	7.66 ± 1.94	0.41
Isoleucine	3.58 ± 1.05	4.33 ± 1.10	0.50
Valine	4.13 ± 1.14	5.04 ± 1.27	0.48
BCAAs	13.84 ± 3.57	17.04 ± 4.3	0.45
Relative (mg/kg/d)			
Leucine	68 ± 10	100 ± 19	0.13
Isoleucine	40 ± 9	56 ± 11	0.18
Valine	46 ± 9	66 ± 13	0.16
BCAAs	154 ± 28	221 ± 42	0.15

Values mean ± SD

Muscle Analyses

Greater gene expression is indicated by lower ΔCt values. Muscle MuRF1 expression was greater in AP versus OP on the day of surgery (1.10 ± 0.00 vs 1.14 ± 0.01, p = 0.03) (**Table 3.5**). Expression of other genes associated with protein breakdown (MAFbx), myogenesis (MyoD, Myogenin, Pax7, Myf5, Myf6) and inflammation (TWEAK, Fn14, TNFα-R, IL6-R) were not different between groups ($p > 0.05$). Large effect sizes ($d > 1.0$) were observed for TNFα-R, IL6-R, MyoD, and MAFbx. A post hoc power analysis (assuming 80% power) showed that sample sizes would have to increase to 10 to 20 individuals for group differences of these proteins to reach statistical significance at the 0.05 level.

Table 3.5. Gene expression and effect sizes for markers of inflammation, myogenesis, and protein breakdown in AP versus OP on day of surgery

Gene	AP (ΔCt)	OP (ΔCt)	p-value	Effect Size	Sample Size Estimate
Inflammation					
TWEAK	1.17 ± 0.13	1.25 ± 0.01	0.32	0.80	42
Fn14	1.18 ± 0.02	1.16 ± 0.03	0.60	0.56	102
TNFα-R	1.14 ± 0.01	1.10 ± 0.02	0.11	2.17	10
IL6R	1.13 ± 0.00	1.14 ± 0.01	0.23	1.56	16
Myogenesis					
MyoD	1.14 ± 0.02	1.11 ± 0.02	0.33	1.08	18
Myogenin	1.12 ± 0.06	1.16 ± 0.02	0.35	0.86	46
Pax7	1.18 ± 0.03	1.17 ± 0.01	0.83	0.18	926
Myf5	1.20 ± 0.10	1.24 ± 0.02	0.50	0.60	92
Myf6	1.12 ± 0.03	1.11 ± 0.03	0.77	0.28	416
Protein Breakdown					
MAFbx	1.05 ± 0.00	1.07 ± 0.02	0.33	1.21	20
MuRF1	1.10 ± 0.00	1.14 ± 0.01	0.03	3.83	6

Skeletal muscle mRNA expression of genes associated with inflammation, myogenesis and protein breakdown were normalized to the geometric mean of β-actin and β2 microglobulin to obtain Δ cycle threshold (ΔCt) values that are shown as mean ± SD for each group

Phosphorylation status and total protein content of proteins in inflammatory (NFκB, IκBα, p38 MAPK), synthetic (rpS6), and proteolytic (FOXO1) pathways were similar between groups (**Table 3.6**). There was also no difference between groups in total protein content of MyoD and Myogenin. Large effect sizes were noted for p-NFκB p65 (d = 1.01), p-p38 MAPK (d = 1.93) and MyoD (d = 1.37), with corresponding sample sizes of 34, 12, and 20, respectively, estimated for group differences to reach statistical significance at the 0.05 level.

Table 3.6 Protein expression and effect sizes for markers of inflammation, myogenesis, protein synthesis, and protein breakdown in AP versus OP on day of surgery

Protein	AP	OP	p-value	Effect Size	Sample Size Estimate
Inflammation					
p-NFκB p65	2.02 ± 0.04	1.58 ± 0.62	0.41	1.01	34
Total NFκB p65	0.40 ± 0.04	0.71 ± 0.57	0.52	0.77	56
p-IκBα	0.34 ± 0.31	0.41 ± 0.10	0.74	0.28	394
Total IκBα	0.61 ± 0.18	0.71 ± 0.37	0.76	0.34	278
p-p38 MAPK	0.55 ± 0.09	1.07 ± 0.37	0.16	1.93	12
Total p38 MAPK	1.43 ± 0.20	1.31 ± 0.16	0.51	0.65	78
Myogenesis					
MyoD	1.97 ± 0.60	2.60 ± 0.22	0.18	1.37	20
Myogenin	0.98 ± 0.35	1.05 ± 0.35	0.83	0.21	712
Protein Synthesis					
p-rpS6	1.57 ± 1.31	2.74 ± 1.91	0.51	0.71	64
Total rpS6	0.52 ± 0.69	0.64 ± 0.94	0.89	0.15	1478
Protein Breakdown					
p-FOXO1	0.13 ± 0.04	0.35 ±0.43	0.55	0.70	68
Total FOXO1	0.96 ± 0.14	0.92 ± 0.15	0.77	0.30	364

Values mean ± SD

Relationship between inflammation, myogenesis, and recovery outcomes

A strong correlation was noted between Pax7 expression on the day of surgery and week 2 quadriceps circumference (**Fig. 3.2 A**, r = -0.97, p = 0.03). Week 4 quadriceps circumference was also associated with Fn14 expression at the time of ACL reconstruction (**Fig 3.2 B**, r = 0.997, p =0.003). A correlation between TNFα-R expression and week 8 quadriceps circumference was also observed (**Fig. 3.2 C**, r = -0.96, p = 0.04).

Quadriceps strength at week 8 was strongly correlated with the expression of Myogenin (**Fig. 3.4 A**, r = -0.99, p = 0.01), Myf5 (**Fig. 3.4 B**, r = -0.97, p = 0.03), TWEAK (**Fig 3.3 C**, r = -0.97, p = 0.03), and p-IκBα (**Fig 3.3 A**, r = -0.96, p = 0.04). There was also a trend for the association of TNFα-R and week 8 strength (**Fig 3.3 B**, r = 0.94, p = 0.056).

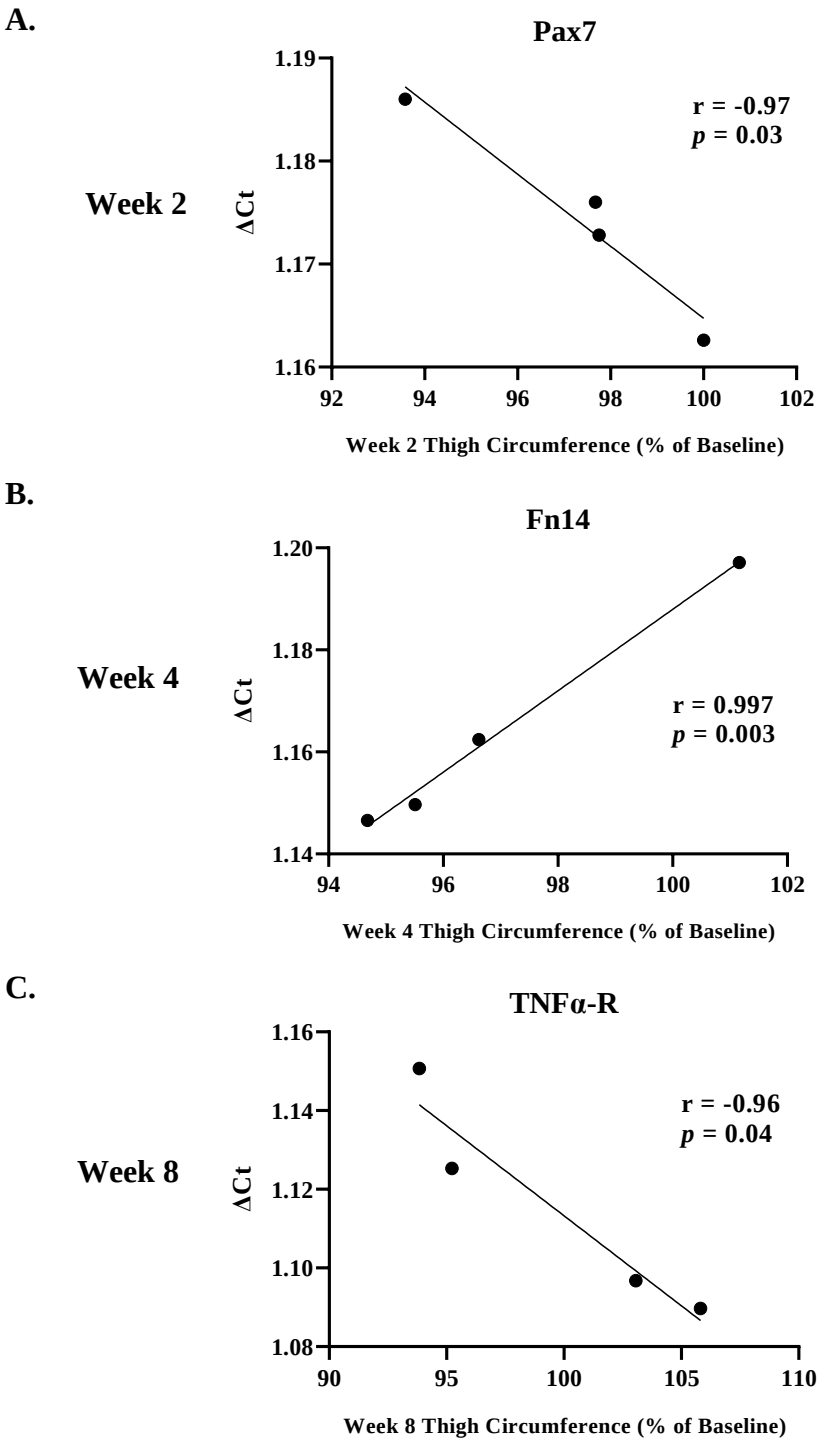

Figure 3.2. Association of perioperative inflammation and myogenic regulatory factor expression with postoperative thigh circumference. Skeletal muscle mRNA expression of paired box 7 (Pax7) (A), fibroblast growth factor-inducible 14 (Fn14) (B), and tumor necrosis factor-α-receptor (TNFα-R) (C) were normalized to the geometric mean of β-actin and β2 microglobulin to obtain Δ cycle threshold (ΔCt) values. Note that lower ΔCt values indicate greater gene expression. Quadriceps circumference at week 2, 4, and 8 are shown relative to baseline.

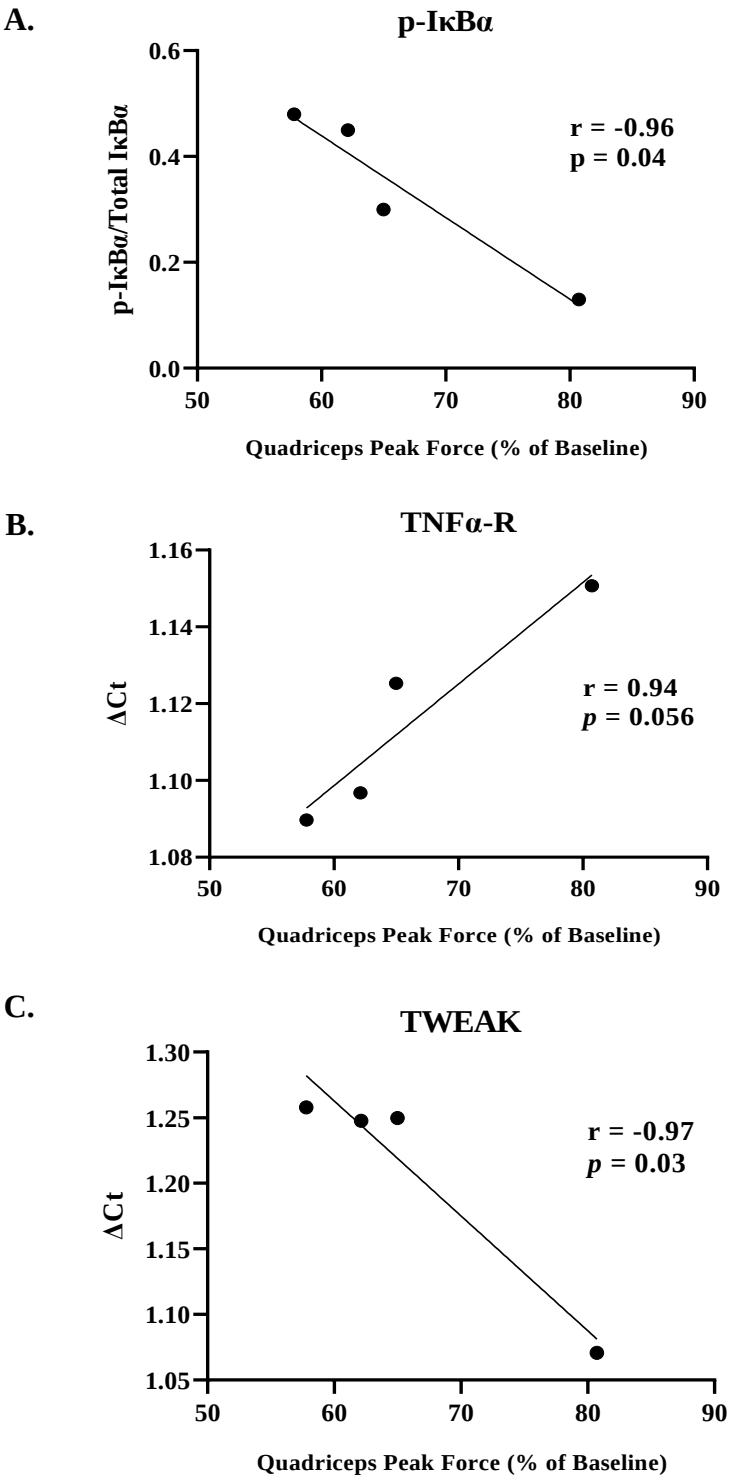

Figure 3.3. Association of perioperative muscle inflammation with week 8 quadriceps strength. Skeletal muscle mRNA expression of tumor necrosis factor-α-receptor (TNFα-R) (B), and TNF-like weak inducer of apoptosis (TWEAK) (C) were normalized to the geometric mean of β-actin and β2 microglobulin to obtain Δ cycle threshold (ΔCt). Note that lower ΔCt values indicate greater gene expression. p-IκBα protein abundance (A) is shown relative to Total IκBα. Week 8 quadriceps peak isometric force is shown relative to baseline.

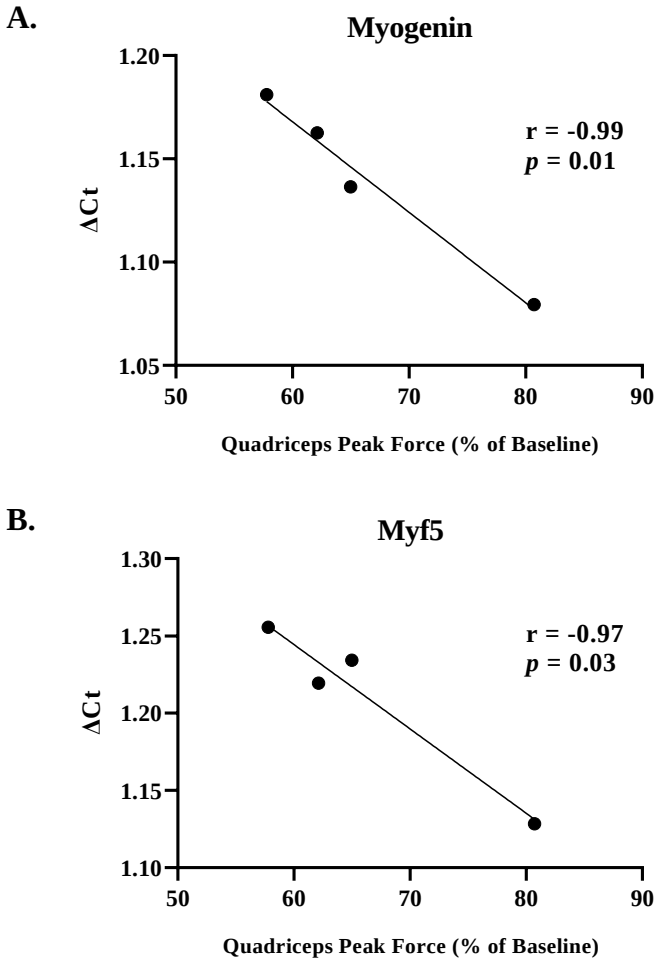

Figure 3.4. Association of perioperative myogenic regulatory factor expression with week 8 quadriceps strength. Skeletal muscle mRNA expression of Myogenin (A) and myogenic factor 5 (Myf5) (B) were normalized to the geometric mean of β-actin and β2 microglobulin to obtain Δ cycle threshold (ΔCt) values. Note that lower ΔCt values indicate greater gene expression. Week 8 quadriceps peak isometric force is shown relative to baseline.

Discussion

The current study assessed the influence of protein intake on perioperative inflammatory and myogenic signaling in vastus lateralis muscle, and if these signaling pathways were related to postoperative muscle recovery outcomes. Lower expression of static markers of proteolysis in the OP group at the time of surgery suggest muscle protein breakdown (MPB) was attenuated in

these individuals While markers of inflammation and myogenesis were not different between groups, a post hoc power analysis suggests sample sizes likely limited significance of these results. Most notably, inflammation and myogenic regulatory factor expression at the time of surgery are seemingly associated with postoperative thigh circumference and quadriceps strength during postoperative rehabilitation.

Bamman et al. (15) were the first to suggest that muscle inflammation at the time of surgery may influence postoperative recovery outcomes after identifying a state of heightened basal proinflammatory signaling in muscle surrounding the diseased hip of some total hip arthroplasty (THA) patients. Dichotomizing patients using median expression of the TWEAK receptor fibroblast growth factor-inducible 14 (Fn14) into groups considered susceptible ($MuIS^{(+)}$; high Fn14 expression) or not susceptible ($MuIS^{(-)}$; low Fn14 expression) to excessive muscle inflammation revealed $MuIS^{(+)}$ individuals had heightened expression of all inflammatory genes evaluated (e.g., TNFα, IL-6, TWEAK) and decreased muscle protein synthesis (MPS) in muscle surrounding the diseased hip. The heightened inflammatory signaling and changes in MPS in the $MuIS^{(+)}$ versus $MuIS^{(-)}$ group would be expected to limit postoperative recovery by reducing muscle regenerative capacity and inducing muscle atrophy. Heighted inflammatory cytokine expression in muscle surrounding the knee after ACL injury may similarly limit postoperative muscle recovery potential. Attenuating intramuscular inflammatory signaling prior ACL reconstruction may therefore protect muscle mass and enhance recovery in the postoperative period.

Dietary protein intake was considered as a countermeasure to excessive skeletal muscle inflammation in the current study given the hypothesized antioxidant potential of BCAAs. Findings from cell and animal models suggest that an abundance of BCAAs may improve redox

protection and attenuate oxidative-stress related inflammatory signaling given these amino acids' role as precursors for the intracellular antioxidant glutathione (GSH) (18). Treating C2C12 muscle cells with BCAA-rich whey protein, for example, increased GSH levels by 25.7 % and 138% at low and high concentrations, respectively (19). Dietary protein may also modulate satellite cell dynamics directly by increasing their activity (i.e., number proliferating cells) (27, 28) and abundance (16, 17). We therefore hypothesized that dietary protein intake of 2.0 versus 1.0 g protein·kg^{-1}·d^{-1} for two weeks prior to ACL reconstruction would optimize postoperative recovery potential by attenuating inflammation and promoting satellite cell abundance and/or activity.

In contrast to study hypotheses, inflammation and myogenic regulatory factor expression were not different between groups in the current study. Low statistical power due to the small sample size (n=5) may have limited significance of these results. A large effect size (d > 1.0) was noted for gene expression of TNFα-R, IL6-R, and MyoD and for protein abundance of p-NFκB p65, p-p38 MAPK, and MyoD. A post hoc power analysis (power set at 0.80) revealed that a total sample size of 10 to 18 subjects would be needed (n=34 for p-NFκB p65) for group differences of these inflammatory and myogenic markers to reach statistical significance at the 0.05 level. The possibility exists that a significant treatment effect would have been observed with a larger sample size. Insignificant findings may also be due to the diet intervention itself. Prescribing higher protein intake and incorporating beef into meals of the OP diet was intended to provide a greater abundance of dietary EAAs (BCAAs in particular) versus what was consumed in the AP group. The lack of differences in absolute and relative amounts of BCAAs between AP and OP individuals may be why the proposed effect of these amino acids on inflammation and myogenesis did not differ between groups.

While inflammatory and myogenic signaling appear unaltered by different levels of protein intake, the intervention may have attenuated muscle protein breakdown (MPB) in the OP group. Kim et al. (29) have shown that a greater whole body net protein balance following consumption of a high (70 g) versus moderate (40 g) protein meal can be attributed to a larger reduction in protein breakdown with the 70 g dose. They hypothesized that greater protein intake and the associated abundance of exogenous amino acids reduced the reliance on protein breakdown to provide amino acid precursors for sustaining protein synthesis. Additional work from our laboratory hypothesized that an observed decrease in post-exercise fractional synthetic rate following a habitual increase in dietary protein intake was due to an abundance of free amino acids that attenuated proteolysis during exercise (30).

Expression of the muscle-specific ubiquitin ligases muscle ring finger 1 (MuRF1) and muscle atrophy F-box (MAFbx)/atrogin-1 were evaluated in the current study to give some insight into whether higher protein intakes influenced MPB. Muscle MuRF1 expression was greater in AP versus OP individuals on the day of surgery ($p = 0.03$), suggesting an abundance of exogenous amino acids in the OP group may have attenuated breakdown in the fasted state during ACL reconstruction. Although a similar effect was not significant for MAFbx, the calculated effect size ($d = 1.21$) and subsequent power analysis revealed a sample size of 20 would be needed for the group difference to reach statistical significance at the 0.05 level. While these findings suggest breakdown was lower in the OP group, this must be interpreted with caution given that average caloric intake relative to body weight tended to be higher ($p=0.08$) in the OP versus AP group suggesting the alterations in proteolytic gene expression may not be the result of protein intake alone.

Although MPB appeared lower during surgery in OP versus AP individuals, postoperative changes in thigh circumference and strength were not different between groups (data not shown). Extending the Bamman et al. (15) proposal that inflammation at the time of surgery dictates regenerative capacity and postoperative recovery, we evaluated whether perioperative markers of inflammation and myogenesis were associated with recovery outcomes after ACL reconstruction. We admit that the small sample size (n=4) and correlative nature of this analysis are limitations and consider the findings preliminary and meriting future work. Regardless, lower expression of the myogenic regulatory factor Pax7 and higher levels of Fn14 at the time of surgery were associated with a greater loss of thigh circumference at week 2 and week 4, respectively. Indeed, these findings correspond with the Bamman study (15) that suggested greater Fn14 expression in TKA patients would decrease regenerative capacity and limit postoperative recovery potential. In contrast to what was expected, higher levels of TNFα-R at the time of surgery were associated with greater thigh circumference at week 8. This contradictory finding may reflect the dual roles of inflammatory signaling in both promoting (31) and inhibiting (11) myogenesis, or may be a result of the time elapsed from surgery. Disuse in the early postoperative period (< 1 month) leads to a loss of muscle mass that is subsequently restored as rehabilitation incorporates more intense exercises and workloads. By two months post-ACL reconstruction in the current study, thigh circumference was increased above baseline values in two subjects while deficits persisted in the other two. Although this divergent response to rehabilitation may be related to TNFα-R expression at the time of surgery, it's possible that other factors such as training status or gains in fat mass influence changes in thigh circumference during later phases of rehabilitation.

Perioperative markers of inflammation and myogenesis were also associated with quadriceps strength at week 8. Individuals with lower expression of the myogenic regulatory factors myogenin and Myf5 at the time of surgery, for example, had less quadriceps strength at week 8 relative to baseline values. Greater day of surgery expression of TNFα-R and p-IκBα, a regulator of pro-inflammatory NFκB signaling, was also associated with lower quadriceps strength at week 8 relative to baseline. These findings collectively suggest that heightened intramuscular pro-inflammatory signaling and a corresponding downregulation of myogenic regulatory factor gene expression at the time of surgery are associated with negative functional outcomes postoperatively. TWEAK expression does not follow this pattern, however, as increased expression of this cytokine was associated with greater week 8 strength relative to baseline. Although this may reflect the divergent roles of this cytokine in both stimulating (32) and inhibiting (8, 10) myogenesis, it also appears that this correlation is driven by one outlier. Expression of TWEAK and quadriceps strength are both greater for 1 person, however, TWEAK expression was the same for the other three subjects despite differences in strength.

In conclusion, observing a significant effect of preoperative protein intake on muscle inflammation and myogenic regulatory factor expression was likely limited by the small sample size (n=5). Protein breakdown, however, did appear attenuated at the time of surgery in the OP group. While it's possible this would protect muscle mass in OP individuals, thigh circumference or strength were not different between groups in the postoperative period. Greater inflammatory cytokine expression and diminished markers of myogenesis at the time of surgery were generally associated with a greater loss of thigh circumference and lower strength at week 8. These findings provide preliminary evidence suggesting perioperative muscle

inflammation and regenerative capacity are associated with postoperative muscle recovery outcomes.

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CHAPTER 4

Optimal protein and feeding strategies to accelerate muscle mass and functional recovery from anterior cruciate ligament reconstruction: A case study

Introduction

Anterior cruciate ligament (ACL) injury and subsequent surgical reconstruction disrupts normal limb function and mobility. The associated decline in activity and deficits in neuromuscular signaling induce a rapid loss of muscle mass (i.e., disuse atrophy) and function given the unloading and reduced neural activation of muscle. Disuse-related muscle atrophy and weakness following ACL injury and reconstruction can be challenging to overcome, and often persists after physical rehabilitation efforts (1). Failing to reverse these deficits in muscle mass and functional capacity may limit activities of daily living, physical performance, and quality of life. Developing strategies to attenuate muscle atrophy and preserve muscle function with disuse are therefore necessary to accelerate and optimize recovery from ACL reconstruction.

Muscle mass is regulated by rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) that fluctuate throughout the day in response to feeding and fasting. A persistent negative net balance (MPS < MPB) resulting from declines in MPS and a possible early increase in MPB underlies decreases in muscle mass during conditions of disuse (2). Interventional strategies designed to attenuate this decline in MPS (postabsorptive and postprandial) and possible early increase in MPB would therefore protect muscle mass under disuse conditions. Manipulating the amount and quality of protein consumed has been studied in experimental models of disuse (i.e., bed rest and immobilization in healthy individuals) as a way to increase the anabolic response to protein ingestion, overcome deficits in postprandial MPS, and preserve muscle mass (3-6). However, this concept has rarely been extended to disuse

conditions after orthopedic surgery that are also accompanied by some muscle activation during postoperative rehabilitation. Given that exercise sensitizes skeletal muscle to the anabolic effect of protein ingestion (7), early rehabilitation may act synergistically with a protein-based intervention to overcome disuse-related deficits in protein turnover and protect muscle mass. The synergistic effect of physical rehabilitation (i.e., resistance exercise) and protein intake on increasing MPS may result in net muscle protein accretion and hypertrophy of muscle when performed habitually.

Research focused on the synergistic effect of exercise and protein intake on rates of MPS generally implements resistance exercise at workloads much greater than what is possible after injury. Whether the body weight or light-load resistance exercise and electrical stimulation of muscle during early rehabilitation has a stimulatory effect on rates of MPS or improves anabolic sensitivity to protein ingestion is not completely understood. Whether standard rehabilitation combined with a protein-based diet intervention would attenuate disuse atrophy and accelerate recovery of muscle mass and function in the postoperative period after ACL reconstruction is unclear. This case study characterizes changes in quadriceps strength and thigh circumference of the injured and non-injured leg during standard rehabilitation from ACL reconstruction in a patient consuming ~2.0 g protein·kg^{-1}·d^{-1}. The aim of this preliminary study is to aid in the development of future research and the design of optimal protein-based diet interventions to attenuate muscle atrophy and weakness and accelerate recovery outcomes after musculoskeletal injury or orthopedic surgery.

Patient overview and surgery details

The patient was a 27 year old male undergoing his first ACL reconstruction surgery. He was 68 kg and 175 cm (22.2 kg/m^2 BMI) at the time of surgery, and reported doing some form of

cardio exercise twice a week (30 – 60 mins) before the injury. The patient had his ACL reconstructed using a bone-tendon-bone (BTB) autograft 36 days after the injury occurred. He was initially anesthetized with propofol and then maintained with inhalation anesthetic (sevoflurane) during surgery. The patient also received an adductor canal and geniculate nerve block using 0.25% Marcaine with 1:200,000 epinephrine. A pneumatic tourniquet was placed 'as-high-as-possible' on the thigh of the injured limb during sterile preparation and draping, and inflated to 250 mm Hg prior to incision and until the graft was harvested (~14 minutes).

Postoperative rehabilitation

The patient used crutches and his knee was immobilized in a leg brace locked at ~0° of flexion until the first postoperative visit with the physician (< 7 days). The patient was then cleared to progress with weight bearing as tolerated (WBAT) and to attend twice weekly physical therapy. All rehabilitation was done with measures of progress performed by by the same physical therapy assistant and following the Orthopedic Associates of Hartford return-to-sport after ACL reconstruction protocol (**Appendix I**) and phase-specific exercises (**Appendix II**). Initial rehabilitation focused on controlling pain, reducing swelling and restoring normal range of motion. An early emphasis on WBAT, isometric exercises, and electrical stimulation of muscle through neuromuscular electrical stimulation (NMES) was also done to facilitate the return of normal neuromuscular signaling. Rehabilitation then shifted to incorporate progressive resistance exercise (i.e., body weight then higher loads) after neuromuscular control and range of motion was restored. Plyometric and agility exercises were also incorporated into later phases of rehab. A summary of phase-specific goals and exercises are shown in **Table 4.1**.

Table 4.1 Overview of the postoperative ACL rehabilitation protocol

	Week 1	Week 2-4
Goals:	Restore knee extension, gradually increase knee flexion to 90°; diminish pain and swelling; restore patellar mobility; reestablish quadriceps control; improve ambulation to full weight bearing.	Maintain knee extension; gradually increase knee flexion to 110°-120°; diminish pain and swelling; maintain patellar mobility; restore proprioception; increase muscular training.
Exercises:	• Ankle pumps • Ankle resistance band exercises • Quadriceps and gluteal set exercises • Straight leg raises • NMES	• NMES • OKC knee extension (90°- 40°, no weight) • Quadriceps isometrics (no weight) • Single leg raises • Front and lateral step-ups • Lateral step overs • Mini squats (0°-30° flexion, DL, BW) • Leg press (DL, sub-maximal)
	Week 4-8	**Week 8-12**
Goals:	Quadriceps strength to >75% of non-injured leg; restore knee ROM to 0°-125°; improve lower extremity strength and endurance; enhance proprioception and neuromuscular control.	Quadriceps strength to >80% non-injured leg; restore full knee ROM, normal gait pattern; continue to improve lower extremity strength balance, proprioception, neuromuscular control, and muscular endurance.
Exercises:	• OKC knee extension (90°- 40°, no weight) • Quadriceps isometrics (no weight) • Single leg raises • Front and lateral step-downs • Lateral step overs • Mini Squats (0°-50° flexion, DL) • Leg press (DL and SL, increasing resistance) • Perturbation training (DL and SL) • Eccentric exercises • Core strengthening	• Progress intensity and duration of exercise performed in the previous phase • Squats (DL and SL, internal and external cues) • SL anterior reach • Plyometric leg press • Progress step-down and lateral step exercises with resistance bands • Bridges with knee extension (DL and SL) • Anterior and lateral lunges • Perturbation training • Eccentric exercises
	Week 12-16	
Goals:	Quadriceps strength to <15% difference between legs; increase muscle power, endurance and neuromuscular control; begin selected skill drills.	
Exercises:	• Progress intensity and duration of exercises performed in the previous phase • Squats (DL and SL, internal and external cues) • Lateral and forward squat walks with resistance band • Side and prone planks (with leg raises) • Plyometric training protocol • Begin walk-to-run protocol • Eccentric exercises • Agility exercises	

Anterior cruciate ligament (ACL), body weight (BW), double leg (DL), neuromuscular electric stimulation (NMES), open kinetic chain (OKC), range of motion (ROM), single leg (SL).

Diet intervention

The patient's energy requirements were established relative to estimated resting energy expenditure (Harris-Benedict equation), estimates of energy intake, and level of physical activity reported at baseline. This information was used to develop a 1-week, eucaloric (i.e., weight maintenance) cycle menu that provided 2.0 g protein·kg^{-1}·d^{-1}, 30% of energy intake from fat, and remaining calories from carbohydrates. The diet intervention also incorporated 5 servings (3 oz) of beef per week as a high quality protein source (see **Table 4.2** for example day). The patient followed this prescribed diet 2 weeks prior to ACL reconstruction and continued the diet intervention for 16 weeks postoperatively. A post-rehabilitation supplement was also integrated into the overall diet design (see **Table 4.2** for example day). The patient consumed 1 serving (~27.4 g) of IsoPrime Beef™ directly after each physical therapy appointment. The macronutrient and amino acid composition of the supplement was the same as previously reported in Chapter 3 (**Table 3.1**).

Table 4.2 Example of dietary meal plan provided to patient

General		Day with Rehabilitation	
Breakfast	1 bagel with 2 Tbsp. of nut butter, 2/3 cup low-fat flavored yogurt, 1 banana	**Breakfast**	1 bagel with 2 Tbsp. of cream cheese, 2 oz. turkey sausage, 8 oz. lemonade
Morning Snack	5 Triscuit crackers, ¾ cup of 2% cottage cheese	**Morning Snack**	1 large apple with 2 Tbsp. of peanut butter, 2/3 cup of low-fat flavored yogurt
Lunch	1 spicy black bean burger with 1 slice of cheese on spinach tortilla, 1 cup of baby carrots, 8 oz. lemonade	**Lunch**	1 large tortilla wrap, 1/3 cup hummus, 1 cup assorted non-starchy vegetables, 1 cup of baby carrots with 1 Tbsp. of ranch on the side, ½ cup of apple sauce
Dinner	3 oz. of grilled sirloin tips, 1 cup of rice, ½ cup cooked green beans	**After Rehabilitation**	1 small orange, 1 serving IsoPrime Beef™ supplement
Evening Snack	2/3 cup low-fat flavored yogurt, 1 banana	**Dinner**	Fajita – two 8-inch tortilla wraps, 4 oz. of cooked chicken, 1 cup of cooked peppers, 2.5 tbsp. guacamole

The patient recorded his food intake for 7 days beginning 1 week prior to surgery, and again at 4, 8, 12, and 15 weeks postoperatively. Nutritionist Pro™ software (Axxya Systems, LLC, Woodinville, WA) was used to analyze these food records and estimate total energy, carbohydrate, fat, and protein intake levels, as well as branched chain amino acid content of the diet. Average daily energy intake for the duration of the diet intervention was approximately 2500 calories provided by ~2.1 g protein·kg^{-1}·d^{-1}, ~4.2 g carbohydrate·kg^{-1}·d^{-1}, and ~32% of total energy from fat (**Table 4.3**). The patient consumed about 8.6 g of leucine, 4.8 g isoleucine, and 5.6 g of valine per day.

Table 4.3 Dietary intake before surgery and for the duration of the rehabilitation intervention

	Pre	Wk 4	Wk 8	Wk 12	Wk 15	Average
Absolute						
Energy (kcal/d)	2422	2396	2468	2856	2383	2505 ± 198
Carbohydrate (g/d)	322	297	281	298	272	294 ± 19
Protein (g/d)	122	121	134	198	143	144 ± 32
Fat (g/d)	81	90	104	77	84	87 ± 11
Relative						
Energy (kcal/kg/d)	37.7	38.0	37.5	37.2	37.2	37.5 ± 0.3
Carbohydrate (g/kg/d)	4.7	4.4	4.1	4.3	3.9	4.2 ± 0.3
Protein (g/kg/d)	1.8	1.8	2.0	2.9	2.1	2.1 ± 0.4
Fat (g/kg/d)	1.5	1.8	2.3	1.8	1.8	1.9 ± 0.3
Percent Total Energy						
Carbohydrate (%)	53	50	46	42	46	47 ± 4
Protein (%)	20	20	22	28	24	23 ± 3
Fat (%)	30	34	38	24	32	32 ± 4

Averages are mean ± SD

Outcomes of the optimal protein and rehabilitation intervention

Thigh circumference was measured at baseline and weeks 2, 4, 8, 12, and 16 postoperatively. Quadriceps strength was also evaluated at baseline before surgery and weeks 8, 12, and 16 after surgery. Methods for measuring thigh circumference and strength are the same

as previously discussed in Chapter 3. Body weight was also measured and recorded by the patient once a week at approximately the same time, after using the restroom, before eating or drinking, and with minimal or no clothing.

Thigh Circumference

Changes in thigh circumference are shown in **Figure 4.1**. Thigh circumference of the injured leg decreased from baseline by approximately 1 cm at 2 weeks postoperatively in the injured leg, and was 1.7 cm less than the non-injured leg at this time point. Injured-leg thigh circumference was similar to baseline and the non-injured leg by week 4. Values progressively increased after week 4 in both legs such that thigh circumference was ~3.8 and 3.9 cm greater at week 16 than baseline in the injured and non-injured leg, respectively.

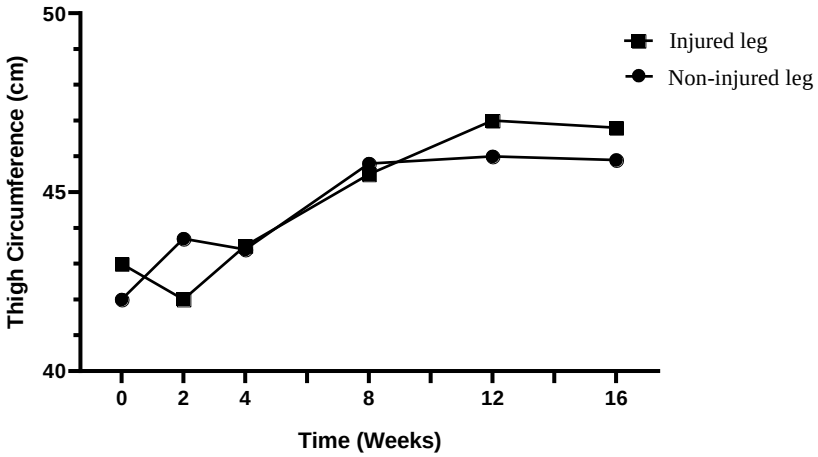

Figure 4.1 Changes in thigh circumference for the duration of diet and rehabilitation intervention for injured and non-injured legs.

Quadriceps Strength

Peak isometric quadriceps force decreased by 26 lb at week 8 compared to baseline in the injured leg (**Fig. 4.2**). While some strength was restored at week 12, strength at week 16 was

lower and similar to week 8. In contrast, peak force of the non-injured leg increased at each time point and was 47.8 lb greater at week 16 versus baseline.

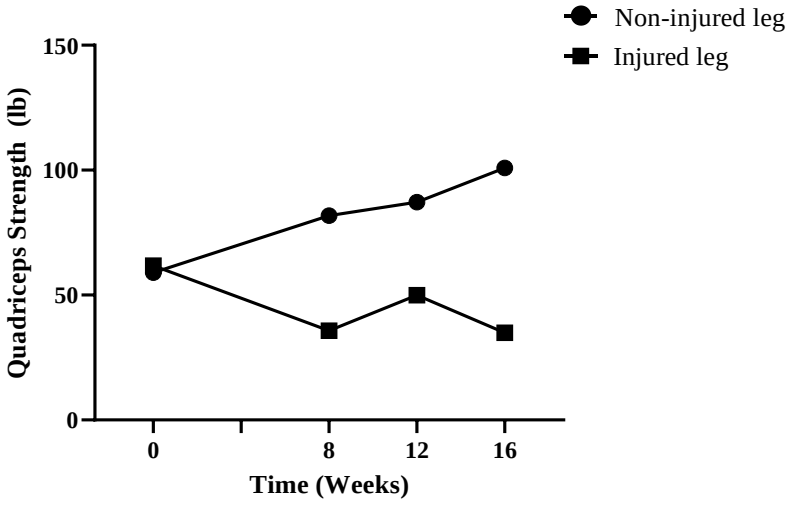

Figure 4.2 Changes in quadriceps strength for the duration of the diet and rehabilitation intervention for injured and non-injured legs.

Body Weight

The subject's initial weight was 148.4 pounds which increased by 6.2 pounds to 154.6 at week 15.

Discussion

While protein-based diet interventions have demonstrated the capacity to protect muscle mass and/or function in experimental models of disuse (i.e., immobilization and bed rest in healthy individuals) (3-6), this concept has rarely been considered in the context of disuse following musculoskeletal injury or orthopedic surgery. The purpose of this case study was to characterize changes in quadriceps strength and thigh circumference after ACL reconstruction in a patient consuming ~2.0 g protein·kg^{-1}·d^{-1} during postoperative rehabilitation. The patient's

gains in injured-leg thigh circumference suggest the diet intervention may have benefited recovery outcomes by attenuating disuse atrophy and potentiating muscle hypertrophy during postoperative rehabilitation. The progressive increase of thigh circumference and quadriceps strength in the non-injured leg also indicates that rehabilitation combined with a diet providing 2.0 g protein$\cdot kg^{-1} \cdot d^{-1}$ has the capacity to promote gains in muscle mass and strength.

Muscle atrophy and weakness occurs rapidly after ACL reconstruction due to injury-related deficits in neuromuscular signaling as well as protective measures implemented to avoid further injury (i.e., immobilization and reduced activity). Quadriceps CSA 16 days post-ACL reconstruction in a group of young patients, for example, was 13.1% lower in the operative leg compared to baseline (8). Quadriceps CSA similarly decreased by 20% during an 11 day postoperative period in another study (9). The loss of thigh circumference observed at week 2 in this case study suggests this patient also experienced a rapid loss of muscle mass in the early postoperative period. The 1.7 cm difference in thigh circumference between legs at week 2 was greater than any other time point. While this difference seems small, anthropometric assessments of whole thigh circumference may be limited in accurately determining muscle losses consequent to quadriceps atrophy. Young et al. (10) found that a 2.5 cm difference in thigh circumference between injured and non-injured limbs corresponded with an approximately 22-33% lower quadriceps cross sectional area (CSA), suggesting that even subtle differences in thigh size can represent a substantial loss of quadriceps mass (11). When interpreting these findings, however, it must be noted that non-injured leg thigh circumference was 2 cm greater at two weeks versus the pre-surgery baseline value. This is may be due to a measurement error at baseline given the similarity between week 2 and week 4 measures (43.7 versus 43.4 cm), the lack of any hypertrophic stimuli from baseline to week 2, and the short time between measures.

Injured-leg thigh circumference was restored and similar to the non-injured leg four weeks after surgery. Thigh circumference also increased during later phases of rehabilitation (i.e., weeks 8, 12, and 16), and was surprisingly ~1 cm greater in the injured versus non-injured leg at 12 and 16 weeks. These findings contrast literature reporting deficits in injured-leg muscle mass that persist during and after physical therapy. Andrade et al. (12), for example, reported a smaller thigh circumference in the injured versus uninjured limb (46.9 ± 4.3 vs. 48.5 ± 3.9 cm, $p < 0.01$) at 4 months post-ACL reconstruction. Quadriceps CSA was similarly 15% lower in the injured versus uninjured leg 6 months postoperatively in another ACL patient population (1).

One explanation for this patient's unexpected return of thigh circumference in the injured leg and increased thigh size in both legs during postoperative rehabilitation may be the diet intervention (i.e., the patient's habitual consumption of higher dietary protein in the context of energy balance). Disuse-related muscle atrophy after ACL reconstruction can be attributed to a decline in MPS (postabsorptive and postprandial) and a possible early increase in MPB (2, 13). Improving the anabolic response to protein ingestion by manipulating the amount and quality (i.e., essential amino acid and leucine content) of protein consumed has been proposed as a way to overcome deficits in postprandial MPS and preserve muscle mass during disuse conditions. Protein intake at the higher end of clinical recommendations may act synergistically with exercise performed during rehabilitation to increase MPS and promote muscle protein accretion when repeated over time.

While literature translating this concept to practice is limited, protein-based interventions do appear to benefit outcomes following orthopedic surgery. Supplementing the diet with essential amino acids (EAAs) after total knee arthroplasty, for example, attenuated the postoperative loss of muscle mass at two (14) and six weeks (14, 15) after surgery. Improving

the anabolic potential of the dietary protein through leucine supplementation also led to greater improvements in thigh circumference (measured 10 cm from the patella) during later phases of rehabilitation after ACL reconstruction (16). These findings suggest that the high quality protein-based diet intervention providing ~2.0 g protein·kg^{-1}·d^{-1} during this patient's postoperative rehabilitation may have similarly attenuated muscle loss and potentiated gains in muscle mass leading to the observed increase in thigh circumference. The simultaneous and consistent consumption of adequate energy also contributes to optimal protein utilization.

While the diet intervention in this case study appeared to protect muscle mass postoperatively, the benefit did not extend to strength outcomes. Deficits in injured-leg strength were observed at all postoperative time points. Although these findings are consistent with previous research showing knee extension strength is diminished at three (17), four (18) and six (1, 17, 18) months post-ACL reconstruction, it's notable that quadriceps strength deficits persisted in this patient despite the increase in thigh circumference. The attending physical therapist noted the patient's apprehension specific to the injured leg during testing at week 16 and greater pain levels compared to previous testing. This may explain why strength was restored at week 12 but then decreased again at week 16. It is possible the diet intervention would have benefited the restoration of strength at time points later than what were evaluated in the current case report. Future research should consider the effect of a protein-based intervention on strength outcomes during early activity and return-to-sport phases of rehabilitation (> 4 months postoperatively).

This case study provides insight into the anabolic potential of exercise during rehabilitation combined with a protein-based diet intervention. The synergistic effect of resistance exercise and protein intake in increasing rates of MPS is generally observed using

workloads much greater than what is possible in the postoperative period after ACL reconstruction. Whether body weight and light-load resistance exercise performed during rehabilitation has a stimulatory effect on rates of MPS or improves the anabolic response to protein ingestion is not fully understood. While strength was not restored in the injured leg in this patient, quadriceps peak force was increased substantially at week 16 compared to baseline in the non-injured leg (85.7 lb vs. 47.9 lb). Non-injured leg thigh circumference also progressively increased at each postoperative time point. This is consistent with previous literature in healthy young men showing 12 weeks of very light load resistance exercise (i.e., 16% of 1-repetition maximum) followed by intake of a protein-containing nutrient supplement led to a 3% hypertrophy of the quadriceps muscle (19). Findings in this individual suggest that rehabilitation acts as a 'resistance training'-type stimulus that, when combined with a diet providing 2.0 g protein$\cdot kg^{-1}\cdot d^{-1}$, can lead to gains in muscle mass and strength.

Observations for this case study must be interpreted with caution given the use of thigh circumference to estimate changes in muscle mass. While the implemented diet intervention was designed for weight maintenance (i.e., ± 5 lbs.), it is well noted that increases in body weight and lean body mass accompany strength training interventions. However, thigh circumference measures cannot differentiate the individual contributions of muscle mass and subcutaneous fat to total thigh size. Given the 6.2 pound increase in body weight from pre-surgery to postoperative week 16, the observed increase in this patient's thigh circumference may reflect alterations in leg fat mass. Future work with larger populations should use DXA, magnetic resonance imaging (MRI), or computed tomography (CT) scans to evaluate changes in muscle volume or cross sectional area specifically. Extended outcome assessments of injured leg

strength should also be considered in future work given that quadriceps peak force was not restored at week 16.

Despite these limitations, this case study provides preliminary evidence consistent with scientific literature and suggests a protein-based diet intervention in the postoperative period after ACL reconstruction may protect muscle mass in the injured leg. The progressive increase in thigh circumference and quadriceps strength in the non-injured leg also indicates that physical rehabilitation is analogous to resistance exercise in promoting gains in muscle mass and strength, particularly when combined with a diet providing 2.0 g protein$\cdot kg^{-1}\cdot d^{-1}$. Future research is warranted on the role of nutrition, higher protein intakes specifically, during physical rehabilitation following orthopedic surgery for optimizing muscle mass, strength, and function outcomes.

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CHAPTER 5

Testosterone supplementation upregulates myogenesis and attenuates proteolytic gene expression after exercise and feeding during severe energy deficit in non-obese, young males

Introduction

The effects of exogenous testosterone administration on lean body mass accretion are well-documented (1-3), and suggest testosterone supplementation may be a viable strategy for preserving muscle mass and functional outcomes in healthy populations exposed to extreme stress. US military personnel, in particular, are subjected to extreme stressors during training and combat operations. High physical demands, sleep deprivation, and sustained periods of severe, unavoidable energy deficit lead to a loss of lean body mass that, in some cases, cannot be overcome by manipulating dietary protein intake alone (4-6). Losses in lean body mass under these conditions generally occur with a concomitant suppression of the hypothalamic-pituitary-gonadal axis (7, 8), and may therefore be attenuated with supplemental testosterone (9, 10).

The anabolic effects of supplemental testosterone have been attributed to a modulation of pathways regulating muscle protein synthesis and breakdown (11) as well as satellite cell and muscle pluripotent stem cell commitment and differentiation (12, 13). Upregulation of anabolic signaling through the mechanistic target of rapamycin (mTOR) pathway (14) and downregulation of ubiquitin proteasome-mediated proteolysis (15) has been observed *in vitro* following testosterone administration in cultured muscle cells. A persistent increase in muscle protein synthesis (MPS) and decrease in muscle protein breakdown (MPB) would lead to lean mass accrual over time. Testosterone-mediated increases in the fusion of activated satellite cells to existing muscle fibers has also been hypothesized to contribute to greater muscle volume (16).

Activation, proliferation, and differentiation of normally quiescent muscle satellite cells and pluripotent stem cells in response to the sequential expression of specific transcription factors (i.e., Pax7, Myf5, MyoD, myogenin, MRF4) generates new multinucleated myotubes. Testosterone supplementation in humans has been shown to increase the number of proliferating satellite cells and expression of myogenin, indicating testosterone promotes cell cycle entry and later stages of myogenesis (12). Testosterone-mediated increases in satellite cell number and their fusion with existing fibers may be regulated through androgen receptor-independent (17), and dependent pathways (13, 18, 19), or through a modulation of upstream pro-inflammatory signaling (20-22). Without question, there are a number of potential mechanisms by which testosterone supplementation may mediate changes in muscle mass.

To our knowledge, the molecular impacts of exogenous testosterone administration during severe exercise- and diet-induced energy deficits remain unexplored. Understanding the effect of testosterone on intracellular signaling pathways regulating muscle mass under these conditions will facilitate the development of androgen-related therapies for mitigating muscle losses during situations of extreme operational stress. The objective of this study was therefore to examine mTOR-mediated anabolic signaling, ubiquitin proteasome-mediated proteolysis, and intracellular markers of myogenesis following 28 days of a severe diet- and exercise-induced energy deficit (~55% deficit) with testosterone supplementation (200 mg testosterone enanthate/wk) or a placebo. Androgen receptor and markers of inflammation were examined as potential upstream regulators of these signaling pathways. Analysis of muscle protein and gene expression was done following a 14-day weight maintenance period and a 28-day energy deficit under fasted, rested conditions and in recovery from exercise and a protein-containing meal. We hypothesized that: 1) testosterone supplementation during energy deficit would modulate

androgen receptor expression and intramuscular inflammation to upregulate myogenic and anabolic (i.e., mTOR) signaling pathways, and decrease proteolysis relative to weight maintenance and 2) the magnitude of this response would be associated with changes in lean body mass during the energy deficit.

Methods

Participants

Participants were part of a larger proof-of-concept, single centre, randomized, double-blind, placebo-controlled trial that assessed the effects of exogenous testosterone administration on changes in body composition with 28 days of a severe exercise- and diet-induced energy deficit (10). Participant eligibility and recruitment details have been reported previously (9). Briefly, 50 young (18–39 y), physically active (≥ 2-d/wk aerobic and/or resistance exercise) men who met age-specific US Army body composition standards (23) and had total testosterone concentrations within the normal physiological range (10.3-34.7 nmol/dL) were recruited locally from the Baton Rouge, LA area. Data in this chapter are presented for 20 of those 50 participants to characterize the intracellular response mediating the greatest gains (TEST, n=10) and reductions (PLA, n=10) in leg lean mass during energy deficit. This study was approved by the Institutional Review Board at the Pennington Biomedical Research Center (PBRC, Baton Rouge, LA) and by the Human Research Protection Office of the US Army Medical Research and Material Command (Ft. Detrick, Fredericksburg, MD). All participants provided written, informed consent and the study is registered with ClinicalTrials.gov asNCT02734238

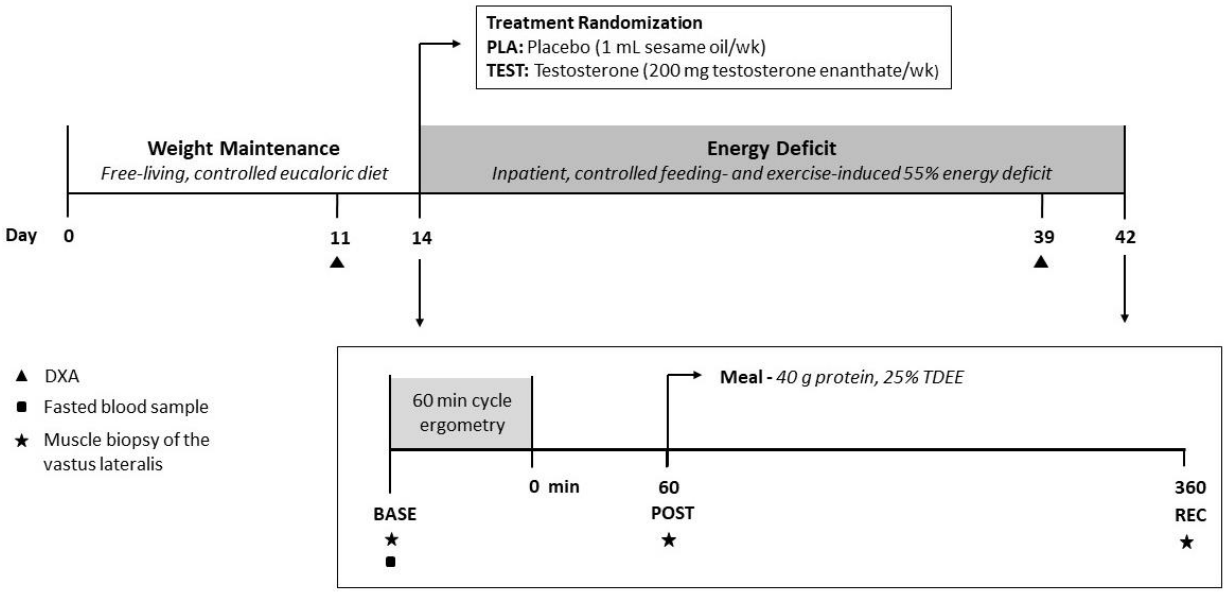


Figure 5.1. Experimental design. The current analysis was part of a larger study assessing the effects of exogenous testosterone administration on changes in body composition after a 28-day diet- and exercise-induced energy deficit designed to be 45% of total energy needs. Muscle biopsies were collected following weight maintenance (WM) and energy deficit (ED) at baseline (Base), 1 h after 60 min of cycle ergometry (Post), and an additional 5 hours into recovery (Rec) from exercise after a protein-containing meal. Steady-state aerobic exercise bouts were matched between WM and ED for each participant based on power output (124 ± 22 W) and total work performed (448 ± 77 kJ).

Experimental design

This study involved 14 days of weight maintenance (WM) followed by 28 days of a highly controlled exercise- and diet-induced energy deficit (ED) (**Fig. 5.1**). Skeletal muscle inflammatory, myogenic, synthetic, and proteolytic signaling pathways were assessed in vastus lateralis biopsies following WM (d 14) and energy deficit (d 42) at baseline and in response to exercise and a protein-containing meal. Participants maintained their habitual physical activity and began a controlled, eucaloric diet providing 1.6 g protein·kg^{-1}·d^{-1}, 30% energy intake from fat, and remaining calories from carbohydrates during the 14-day, free-living WM phase. Energy requirements for weight maintenance were individualized using the Mifflin St Jeor Equation with an activity factor of 1.3, as well as 7-day accelerometer and 3-day activity log data obtained during screening visits (9, 10). Compliance with exercise and diet instructions was

verified by research dietitians, accelerometry, and measuring semi-nude body weight daily with a calibrated digital scale (GSE Inc. Model 450, GSE Scale Systems, Novi, MI) after an overnight fast and morning void (10). Body mass was stable (d 0, 75.6 ± 13; d 14 75.3 ± 13) and dietary intake was similar in both groups during WM (**Table 5.1**).

Table 5.1. Dietary intake during weight maintenance and energy deficit in TEST versus PLA

	TEST (n=10)		PLA (n=10)	
	WM	**ED**	**WM**	**ED**
Absolute intake				
Energy (kcal/d)	2650 ± 131	1784 ± 314	2574 ± 340	1707 ± 268
Carbohydrate (g/d)	344 ± 52	190 ± 39	340 ± 54	188 ± 45
Protein (g/d)	132 ± 25	128 ± 24	121 ± 15	117 ± 16
Fat (g/d)	88 ± 13	60 ± 11	86 ± 11	57 ± 9
Relative Intake				
Energy (kcal/kg/d)	33.5 ± 3.1	25.0 ± 4.0	36.5 ± 3.1	24.0 ± 4.0
Carbohydrate (g/kg/d)	4.4 ± 0.5	2.7 ± 0.5	4.9 ± 0.9	2.6 ± 0.6
Protein (g/kg/d)	1.7 ± 0.1	1.8 ± 0.3	1.70 ± 0.1	1.6 ± 0.2
Fat (g/kg/d	1.1 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	0.8 ± 0.1

Values are presented as mean ± SD. Independent sample t tests were used to compare testosterone (TEST) and placebo (PLA) within weight maintenance (WM) and energy deficit (ED) phases. No differences were observed between groups ($P > 0.05$).

Participants were admitted to an inpatient unit at PBRC following WM to begin the 28-day (days 15 - 42) exercise- and diet-induced ED following randomized (1:1 ratio) at the beginning of this phase to receive weekly intramuscular injections of 200 mg of testosterone enanthate (TEST) or 1 mL of a sesame oil placebo (PLA). This dose of testosterone administered on days 15, 21, 28 and 35 was chosen to maintain normal testosterone concentrations during the severe energy deficit (9). As previously described (10), participants performed approximately 3.5 sessions of varied-intensity (40-85% of predetermined $VO2_{peak}$) aerobic-type exercise per day to increase exercise-induced energy expenditure (EIEE) and elevate total daily energy expenditure (TDEE) by 50% from WM [i.e., ED EIEE = WM EIEE +

(0.5 x WM TDEE)]. The 55% energy deficit was established by setting energy intake at 45% of the elevated TDEE. The macronutrient distribution of the ED diet was similar to WM and dietary intake was not different between groups (**Table 5.1**). Exercise modalities used to increase EIEE have been reported previously (10) and included elliptical, stationary bike, and outdoor/treadmill walking, running, and load carriage (weighted vest ~30% of body mass). Exercise intensity was verified biweekly using open circuit indirect calorimetry (ParvoMedics TruOne 2400, East Sandy, UT) and adjusted when needed to maintain the prescribed EIEE (10). Light calisthenics were also incorporated every 3-4 days but not preformed within 48 hours of exercise testing and muscle biopsies.

Experimental exercise bout and muscle biopsies

Percutaneous muscle biopsies of the vastus lateralis were collected before and after a steady state aerobic exercise bout on day 14 of WM and following the 28-day ED on day 42. The exercise bout included 60 min of cycle ergometry (Lode Excalibur Cycle Ergometer, Netherlands) with exercise intensity matched between ED and WM for each participant based on power output (124 ± 22 W) and total work performed (448 ± 77 kJ). Workloads for the experimental exercise bouts were determined during the first week of WM using intermittent indirect calorimetry assessment of oxygen kinetics throughout a familiarization ride on the cycle ergometer. A total of 3 muscle biopsies were collected from 1 incision on 1 leg per biopsy protocol day using a 5 mm Bergstrom needle with manual suction (15) and under local anesthesia (1% lidocaine). The biopsy needle was inserted at different angles to separate samples sites by ~ 5 cm and limit excessive trauma or inflammation. Muscle biopsies were collected under fasted, rested conditions [Baseline (Base)] and again after the cycle ergometry bout at 60 min (Post) and 360 min [Recovery (Rec)] post-exercise. Participants also consumed a

standardized meal after the 60-min post-exercise biopsy providing 25% of TDEE, 40 g of protein from animal sources, and 30% of kcals from fat. Absolute energy and macronutrient content of this meal was not different between groups during WM or ED (**Table 5.2**).

Table 5.2. Macronutrient composition of the post-exercise meal in TEST versus PLA at weight maintenance and energy deficit

	Testosterone (n = 10)		**Placebo (n=10)**	
	WM	**ED**	**WM**	**ED**
Absolute intake				
Energy (kcal)	676 ± 122	390 ± 58	640 ± 101	376 ± 45
Carbohydrate (g)	81 ± 22	29 ± 10	75 ± 19	26 ± 10
Protein (g)	40 ± 0	40 ± 0	40 ± 0	40 ± 0
Fat (g)	23 ± 4	13 ± 1	21 ± 3	13 ± 1

Values are presented as mean ± SD. Independent sample t tests were used to compare testosterone (TEST) and placebo (PLA) within weight maintenance (WM) and energy deficit (ED) phases. *Indicates between group differences for particular phase ($P < 0.05$)

mRNA expression

Transcript levels of select genes linked to skeletal muscle inflammation [interleukin 6 (IL-6), IL-6 receptor (IL-6R), tumor necrosis factor-α (TNFα), TNFα-receptor (TNFα-R), TNF-like weak inducer of apoptosis (TWEAK), fibroblast growth factor-inducible 14 (Fn14)], myogenesis [MyoD, myogenin, paired box 7 (Pax7), myogenic factor 5 (Myf5)], and protein breakdown [muscle atrophy F-box (MAFbx), muscle ring fiber 1 (MuRF1)] were determined using commercially available TaqMan® probes (Applied Biosystems, Foster City, CA). Total RNA was extracted from ~15 mg of muscle with TRIzol (ThermoFisher, Waltham, MA) and assessed for quality and quantity using a Nanodrop ND-2000 spectrophotometer (NanoDrop, Wilmington, DE). Equal amounts of total RNA (500 μg) were reverse-transcribed into cDNA

using High-Capacity cDNA RT Kits (Applied Biosystems) and a T100^{TM} Thermal Cycler (Bio-Rad, Hercules, CA). Samples were run in 10 µL reactions in duplicate using TaqMan® fast advanced master mix with a Step One Plus Real-Time PCR system (Applied Biosystems). Data were normalized to the geometric mean of GUSB and TUBB mRNA, and fold changes were calculated using the $\Delta\Delta C_T$ method (24). ED data were expressed as a fold change relative to WM for TEST and PLA at each time point (i.e., Base, Post, Rec). One Fn14 value at Base and an IL-6 data point at Base, Post, and Rec were considered outliers and removed given their values were greater than 3 standard deviations from the mean.

Intracellular signaling

Total protein content of androgen receptor (AR) and the relative abundance and phosphorylation state of proteins involved in mammalian target of rapamycin (mTOR)-mediated anabolic signaling were determined using standard SDS-PAGE and Western blot analysis. Approximately 15 mg of muscle were homogenized in ice-cold lysis buffer with protease and phosphatase inhibitors. Homogenized samples were snap frozen in liquid nitrogen, thawed on ice and centrifuged for 15 min at 10,000 g (4°C). Supernatant (lysate) was subsequently collected, and protein concentrations were determined using a 660 nm Protein Assay (ThermoScientific, Rockford, IL). Muscle lysates were solubilized in Laemmli buffer and loaded in equal amounts (i.e., 20 µg/lane) into precast Tris-HCl gels (Bio-Rad). Proteins were then separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad) that were incubated overnight at 4°C with commercially available primary antibodies specific total ribosomal protein S6 (rpS6) (Abcam, Cambridge, MA), p-rpS6$^{Ser240/244}$, total mTOR, p-mTOR^{Ser2448}, total p70 ribosomal protein S6 kinase (p70S6K), p-p70S6K$^{Ser424/Thr421}$, and total AR (Cell Signaling Technology, Danvers, MA). Labeling was performed using horseradish

peroxidase-conjugated secondary antibody (Cell Signaling Technology) and signals were detected using a ChemiDoc XRS system (Bio-Rad) with Image Lab software (Bio-Rad) following application of chemiluminescent reagent (Pierce, Rockford, IL). Heat-shock protein 90 (HSP90) was used to confirm equal amounts of protein loaded per well. Phosphorylation status was expressed relative to the total amount of each protein and total protein content was expressed relative to HSP90. ED data were shown as a fold change from WM for TEST and PLA at each time point (i.e., Base, Post, Rec).

Muscle fiber cross sectional area

A portion of the muscle collected from the WM (d 14) and ED (d 42) Base biopsies was used to characterize cross-sectional area (CSA) of Type II fast-twitch and Type I slow-twitch myosin heavy chain myofibres. Methodological details and findings for all 50 participants have been reported previously (10). Type I and Type II CSA data specific to participants selected for the current chapter have been summarized as a change from WM to ED.

Body composition

Body composition (total body mass, lean body mass, and fat mass) measured via dual-energy X-ray absorptiometry (DXA; Lunar iDXA, GE Healthcare, Madison, WI) was a primary outcome of the parent study (10), and is reported in this chapter for the involved participants as a change from WM to ED. DXA scans were conducted on days 11 and 39 after an overnight fast and morning void using the participant positioning standards reported previously (10).

Endocrine profile

Endocrine profiles were analyzed from fasted blood samples collected before the first muscle biopsy procedure on days 14 and 42, and have been presented previously for all 50 participants (10). In brief, an Immulite 2000 system (Siemens, Llanberis, UK) was used to

analyze blood samples for total testosterone (TT), follicle-stimulating hormone (FSH), estradiol (E2), sex-hormone binding globulin (SHBG), luteinizing hormone (LH), and insulin. Insulin-like growth factor-1 (IGF-1) was analyzed using an enzyme-linked immunoassay (ALPCO, Salem, NH) and Glucose was analyzed on a Beckman DXC 600 Pro (Brea, CA). Blood was sampled between the hours of 06:00 and 09:00 given the diurnal variation of total testosterone in young men (25). Hormone data in the current chapter is presented for the involved participants as a change from WM to ED.

Participant stratification

Baseline inflammatory and myogenic gene expression were initially analyzed for all 50 participants given the previously reported increase in leg lean mass during ED with testosterone supplementation (10), and the positive association between changes in leg lean mass and change in total testosterone following the 28-day energy deficit ($r = .541$, $p < 0.001$). Given that no differences were noted between groups for these parameters (**Fig. 5.2**), the top (TEST) and bottom (PLA) 10 participants with respect to changes in leg lean mass and total testosterone were selected for further analyses (**Fig. 5.3**). This allowed exclusion of participants whose testosterone or leg lean mass did not change during the intervention in an effort to better characterize the intracellular signaling and gene expression mediating the hypertrophic effects of supplemental testosterone during energy deficit.

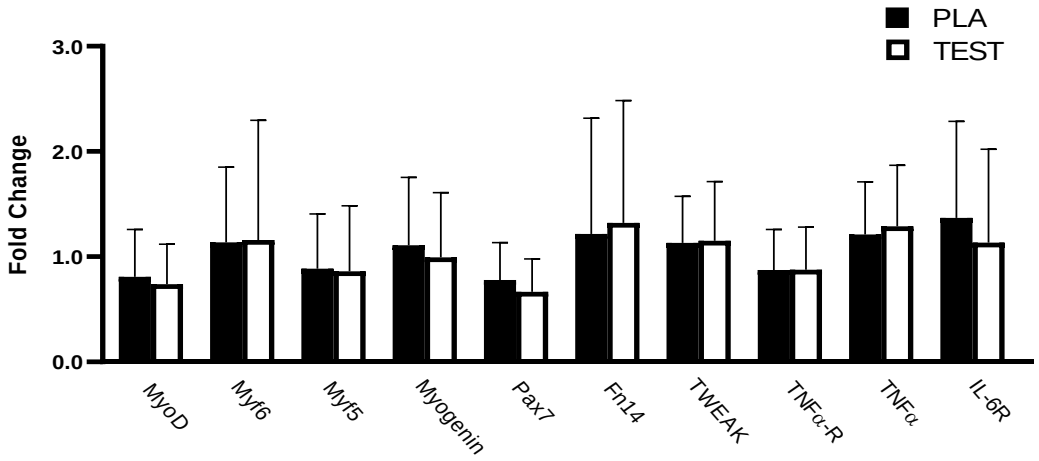


Figure 5.2. Baseline inflammatory and myogenic gene expression for TEST and PLA including all 50 participants. Base gene expression at the end of ED was expressed as a fold change relative to WM for male participants receiving 200 mg of testosterone enanthate [TEST (□)] or 1 mL of a sesame seed oil placebo [PLA (■)] per week during the 28-day ED phase. Data were normalized to the geometric mean of β-actin, and fold changes were calculated using the $\Delta\Delta C_T$ method (24). Unpaired t-tests were used to examine differences between TEST and PLA and values are presented as mean ± SD. No differences were observed between groups ($P > 0.05$)

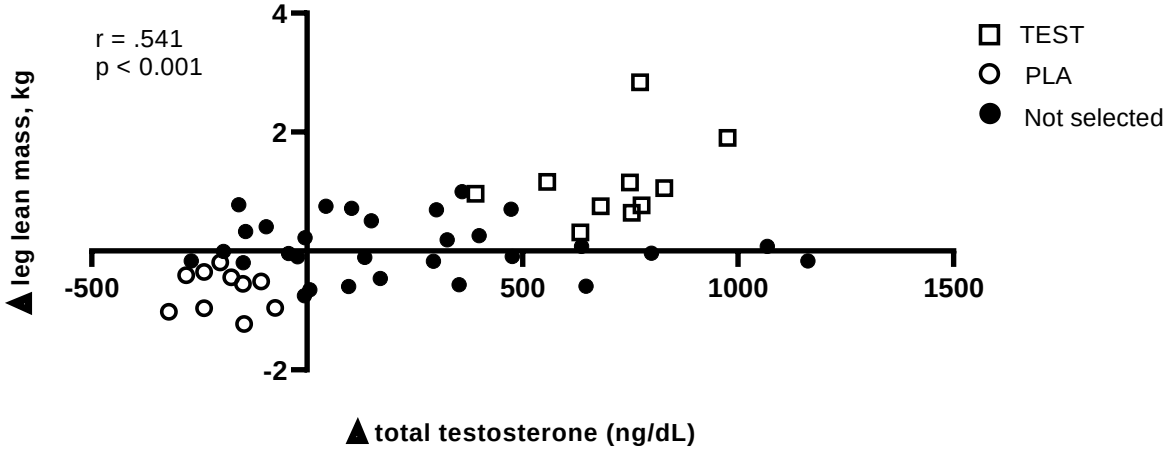


Figure 5.3. Participant stratification according to leg lean mass and total testosterone. Change in leg lean mass (kg) and total testosterone (ng/dL) were positively associated for all 50 participants (r = .541, p < 0.001). We selected the top (TEST) and bottom (PLA) 10 participants (n=20) with respect to these parameters for the gene expression and intracellular signaling analyses included in this chapter.

Statistical analysis

Gene expression and protein abundance data at ED were expressed as a fold change from weight maintenance at Base, Post, and Rec. TEST versus PLA was evaluated at each time point using unpaired *t*-tests. Unpaired *t*-tests were also used to analyze body composition and endocrine profile data, dietary intake, and macronutrient composition of the post-exercise meal in TEST versus PLA. Associations between Fn14 gene expression, AR total protein content, and the change in leg lean mass expressed as ED - WM were explored using stepwise linear regression. Data within the text and figures are presented as mean ± SD. The α level of significance for all statistical tests was two-tailed and set at $P < 0.05$. Data were analyzed using IBM SPSS Statistics were Windows Version 26 (IBM Corp. Armonk, NY, USA).

Results

Participants selected for this analysis were not different with respect to age or BMI (**Table 5.3**) as well as dietary intake during WM and ED, and after the experimental exercise bout (**Table 5.1 and 5.2**). Changes in body composition and endocrine profile in response to the 28-day ED phase, however, were statistically different between groups (**Table 5.4**). The loss of total body and leg mass was less for TEST (-0.9 ± 1.0 kg and -0.3 ± 0.7 kg) versus PLA (-5.1 ± 1.4 kg and -1.8 ± 0.6 kg). The TEST group also displayed a greater preservation and increase in lean body mass (TEST, 3.8 ± 1.2 kg; PLA, -0.9 ± 1.0 kg), leg lean mass (TEST, 1.2 ± 0.7 kg; PLA, -0.7 ± 0.4 kg) and trunk lean mass (TEST, 2.4 ± 0.8 kg; PLA, 0.4 ± 0.7 kg). Testosterone versus placebo supplementation during ED in these participants led to a more positive change in total testosterone (TEST vs. PLA, 711.9 ± 159.3 vs -193 ± 77.8 ng/dL), FT and E2 and a greater

decrease in FSH and LH from WM. A greater increase in SHBG, a marker inversely related to available testosterone, was also observed for PLA.

Muscle TWEAK, TNFα-R, TNFα, IL-6R, IL-6, Myogenin, Pax7, Myf5, and Myf6 gene expression were not significantly different between TEST and PLA at any ED time points (**Fig 5.4 B-F, H-K**). In contrast, Fn14 gene expression at Base following ED was lower in TEST versus PLA (**Fig 5.4 A**, $P < 0.05$). MAFbx and MuRF1 gene expression at Rec following ED was also lower in TEST versus PLA (**Fig 5.4 L-M**, $P < 0.05$), while MyoD gene expression was greater for TEST at the same time point (**Fig 5.4 G**, $P < 0.05$). AR total protein content was greater for TEST versus PLA at the end of ED (**Figure 5.5**, $P < 0.05$). Phosphorylation status and relative abundance of mTOR, p70S6K, and rpS6 were not different between groups at any time point (**Figure 5.6 A-F**).

Stepwise linear regression analyses showed increased androgen receptor total protein content was associated with greater gains in leg lean mass during energy deficit (**Fig 5.6 A**, r = .540, $P < 0.05$). A trend for a similar association between increased leg lean mass and decreased Fn14 gene expression was also observed (**Fig 5.6 B**, r = -.455, $P = 0.058$). Decreased Fn14 gene expression was also associated with increased androgen receptor total protein content (**Fig 5.6 C**, r = -.563, $P < 0.05$)

Table 5.3. Participant baseline characteristics in PLA and TEST

	PLA (n=10)	**TEST (n=10)**
Age (y)	22 ± 3	25 ± 6
Height (cm)	177 ± 7	175 ± 7
Weight (kg)	80 ± 15	71 ± 10
BMI (kg/m^2)	25 ± 3	23 ± 2

Values are mean ± SD.

Table 5.4. Change in body composition, muscle fiber CSA, and endocrine profile with energy deficit

	ED – WM, Δ		
	TEST	PLA	*P-value*
Body composition			
Body mass, kg			
Total	-0.9 ± 1.9*	-5.1 ± 1.4	<0.0001
Lean	3.8 ± 1.2*	-0.9 ± 1.0	<0.0001
Fat	-4.7 ± 1.6	-4.1 ± 1.2	.348
Leg mass, kg			
Total	-0.3 ± 0.7*	-1.8 ± 0.6	<0.0001
Lean	1.2 ± 0.7*	-0.7 ± 0.4	<0.0001
Fat	-1.5 ± 0.4	-1.3 ± 0.3	0.063
Trunk mass, kg			
Total	-0.3 ± 1.4*	-2.2 ± 0.9	0.003
Lean	2.4 ± 0.8	0.4 ± 0.7	<0.0001
Fat	-2.7 ± 1.1	-2.6 ± 1.0	.798
Muscle fiber CSA			
Type I, μm^2	478 ± 1126	394 ± 1141	.876
Type II, μm^2	-827 ± 1151	-1059 ± 1161	.710
Endocrine Profile			
TT, ng/dL	711.9 ± 159.3*	-193.4 ± 77.8	<0.0001
FT, ng/dL	19.0 ± 3.7*	-6.0 ± 1.7	<0.0001
FSH, mIU/mL	-3.2 ± 1.2*	-0.6 ± 1.0	0.007
E2, pg/mL	39.7 ± 17*	-9.1 ± 8.8	<0.0001
SHBG, µg/mL	3.7 ± 9.3*	18.3 ± 9.7	0.003
LH, mIU/L	-2.8 ± 1.0*	-1.1 ± 1.5	0.009
IGF-1, ng/mL	-82.7 ± 85.6	-120.8 ± 77.3	.310
Glucose, mg/dL	-10.2 ± 8.8	-8.5 ± 14.7	.757
Insulin, µIU/mL	-7.4 ± 8.5	-7.0 ± 11.3	.925
Cortisol, µg/dL	4.7 ± 4.3	6.0 ± 4.5	.494

Values are mean ± SD. Data were expressed as energy deficit (ED) minus weight maintenance (WM) and differences between TEST and PLA were analyzed using unpaired t tests. *Indicates group differences ($P < 0.05$) CSA, cross-sectional area; TT, total testosterone; FT, free testosterone; FSH, follicle stimulating hormone; E2, estradiol; SHBG, sex-hormone binding globulin; LH, luteinizing hormone; IGF-1, insulin-like growth factor-1

Inflammation

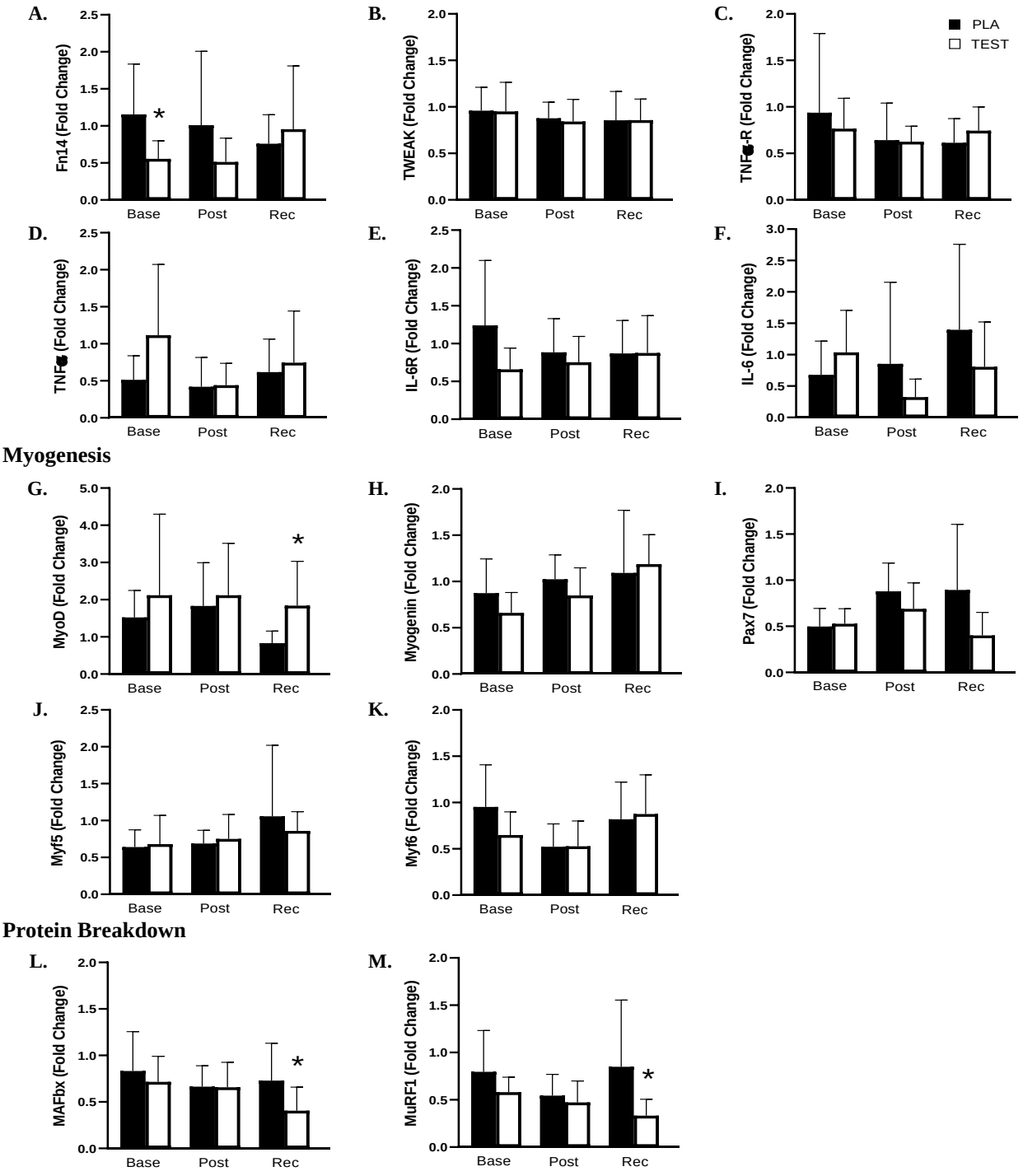


Figure 5.4. Skeletal muscle inflammatory, myogenic, and proteolytic gene expression after energy deficit. ED data were expressed as fold change relative to WM at baseline (Base), 1 h after 60 min of cycle ergometry (Post), and an additional 5 hours into recovery (Rec) from exercise after a protein-containing meal for male participants receiving 200 mg testosterone enanthate [TEST (□)] or 1 mL of a sesame oil placebo [PLA (■)] weekly during the 28-day ED phase. Data were normalized to the geometric mean of GUSB and TUBB, and fold changes were calculated using the $\Delta\Delta C_T$ method. Differences between TEST and PLA at each time point were examined using unpaired t-tests. Values are presented as mean ± SD. *TEST different than PLA, $P < 0.05$. Fn14, fibroblast growth factor-inducible 14; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; TNFα, tumor necrosis factor-α, TNFα-R, tumor necrosis factor-α-receptor; IL-6, interleukin 6; IL-6R, interleukin 6 receptor; Pax7, paired box 7; Myf5, myogenic factor 5; Myf6, myogenic factor 6; MAFbx, muscle atrophy F-box; MuRF1, muscle ring finger 1

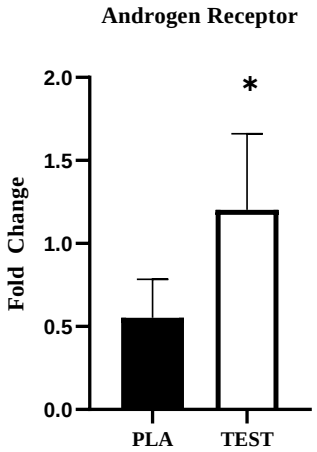


Figure 5.5. Androgen receptor total protein content after energy deficit. Base total protein content of AR at the end of ED was expressed as a fold change relative to WM for male participants receiving 200 mg of testosterone enanthate [TEST (□)] or 1 mL of a sesame seed oil placebo [PLA (■)] per week during the 28-day ED phase. Total protein content was normalized to HSP90 and values are presented as mean ± SD. *TEST different than PLA, $P < 0.05$.

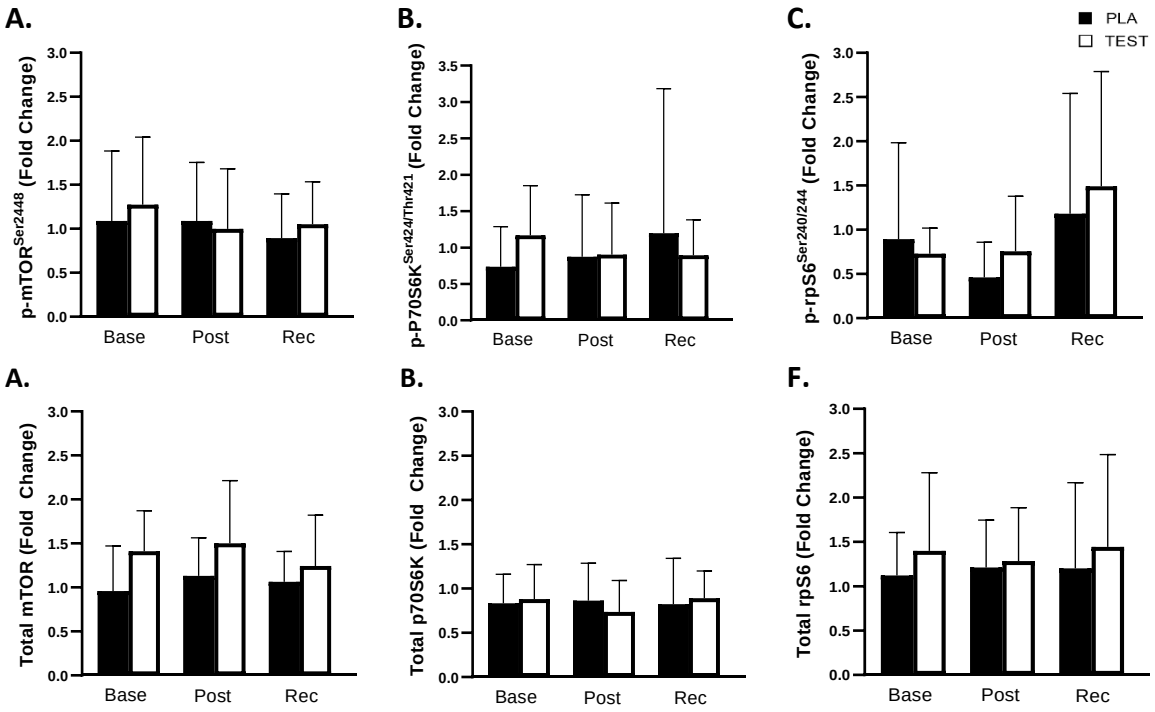
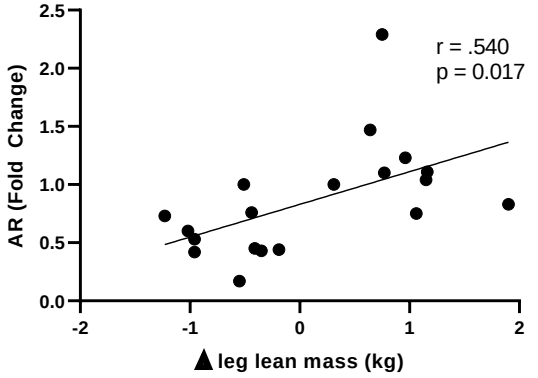
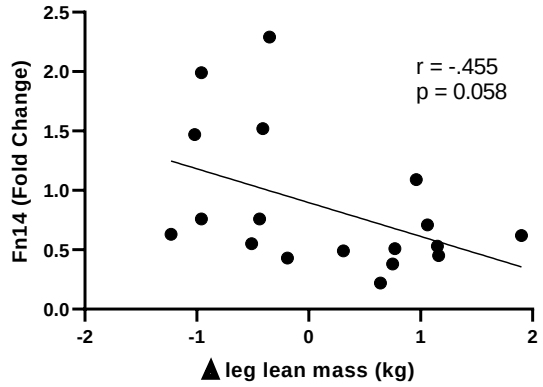


Figure 5.6. mTOR-mediated anabolic signaling after energy deficit. Abundance of p-mTOR^{Ser2448} (A), p-p70S6K$^{Ser424/Thr421}$ (B), p-rpS6$^{Ser240/244}$ (C), total mTOR (D), total p70S6K, and total rpS6 (E) following ED was expressed as fold change relative to WM at baseline (Base), 1 h after 60 min of cycle ergometry (Post), and an additional 5 hours into recovery (Rec) from exercise after a protein-containing meal for participants receiving 200 mg of testosterone enanthate [TEST (□)] or 1 mL of a sesame oil placebo [PLA (■)] weekly during the 28-day ED phase. Phosphorylation status was expressed relative to the total amount of each protein and total protein content was expressed relative to HSP90. Values are presented as mean ± SD and no differences were observed between groups ($P > 0.05$).

A.



B.



C.

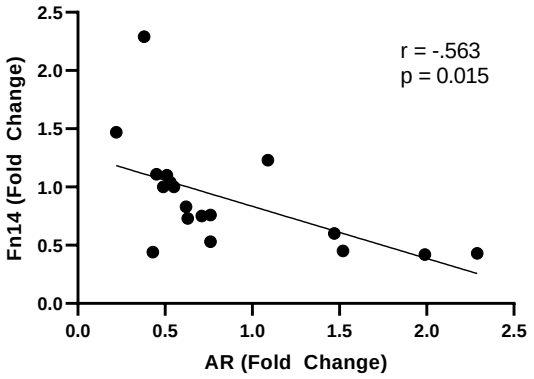


Figure 5.7. Associations between the change in Fn14 gene expression, AR total protein content, and leg lean mass with energy deficit. Leg lean mass measured via DXA is presented as WM – ED (Δ). Fn14 gene expression was normalized to the geometric mean of GUSB and TUBB, and fold change was calculated using the $\Delta\Delta C_T$ method (24). AR total protein content was normalized to HSP90. Fn14 gene expression and AR total protein content following ED were expressed as a fold change relative to WM.

Discussion

The current study examined mTOR pathway signaling, ubiquitin-mediated proteolysis, and myogenic regulatory factor expression following a 28-day diet- and exercise-induced energy deficit (~55% deficit) with supplemental testosterone (200 mg testosterone enanthate/wk) or a placebo. The primary observation of this investigation is that testosterone supplementation during energy deficit attenuated markers of muscle protein breakdown and increased MyoD gene expression at Rec, while mTOR-mediated anabolic signaling was not different between groups at any time point. Base expression of AR and Fn14, two potential upstream regulators of myogenesis and ubiquitin-mediated proteolysis, were different in TEST versus PLA after ED, and were also associated with changes in leg lean mass. These novel findings suggest that exogenous testosterone administration during a severe diet- and exercise-induced energy deficit attenuates proteolysis and enhances myogenesis after exercise and feeding, possibly via AR and Fn14 signaling. This may account, in part, for the increase in leg lean mass observed in the TEST group during ED.

Expression of the myogenic regulatory factor MyoD was greater at Rec in TEST versus PLA after the ED phase, indicating a heightened myogenic response following exercise in these individuals. This is consistent with *in vitro* work (13, 17) and humans studies (12) demonstrating enhanced myogenesis with the administration of exogenous testosterone. The activation, proliferation, and differentiation of satellite cells (i.e., myogenesis) is critical following exercise to repair and replace damaged muscle fibers, and ultimately maintain muscle mass and function (26, 27). Increased fusion of activated satellite cells to existing muscle fibers may also contribute to greater muscle volume (16). This suggests that the observed testosterone-mediated increase in myogenic signaling after exercise and feeding may promote the

maintenance or increase of muscle mass during energy deficit. The decreased expression of muscle protein breakdown markers (i.e., MAFbx and MuRF1) in TEST versus PLA at Rec, indicates testosterone supplementation may also protect muscle mass during energy deficit by attenuating proteolysis after exercise and feeding.

Interestingly, we observed no differences between groups in mTOR signaling, suggesting a limited role of protein synthetic pathways in mediating the hypertrophic effect of testosterone supplementation during energy deficit. This was surprising given literature reporting increased rates of protein synthesis under fasted conditions following exogenous testosterone administration in humans (28, 29). While this discrepancy may be due to differences in energy status (i.e., energy balance vs. energy deficit), the role of mTOR signaling in regulating protein synthesis during basal, non-stimulated conditions is unclear (29). It is possible that fasted rates of synthesis were increased similar to previous studies without concomitant changes in mTOR pathway activity. Future work should employ dynamic measures of protein synthesis (i.e., stable isotope methodology) to further understand the effect of testosterone on muscle protein synthesis during energy deficit.

The lack of changes in mTOR signaling in the TEST group was unexpected given mechanistic studies implicating the mTOR pathway in testosterone-induced increases in myotube hypertrophy (14, 31). However, this *in vitro* work may not accurately reflect the fasted conditions under which testosterone-induced increases in protein synthesis have been observed in humans. Rossetti et al. (11) have suggested that future studies should employ periods of fasting in animal models and serum or nutrient deprivation prior to harvesting cultured cells to better understand molecular signaling underlying the androgen-mediated increase in protein synthesis observed in the fasted state in humans.

Androgen receptors (AR) may mediate some of the effects of supplemental testosterone during energy deficit. We observed greater AR total protein content at the end of the ED phase in TEST versus PLA. Satellite cells are the predominant site of AR expression in human skeletal muscle (32) suggesting the observed increase in androgen binding sites may be linked to the regulation of myogenic activity. Androgens that cross the cell membrane bind the AR, and the resulting complex alters transcriptional activity by interacting with androgen response elements (ARE) of target genes. Lee et al. (18) have reported a relationship between androgen-AR signaling and enhanced myogenin expression and myoblast differentiation following testosterone administration in C2C12 cells. Intact AR was also necessary for the increase in mRNA and protein levels of MyoD and myosin heavy chain II (MHC) in mouse mesenchymal C3H 10T1/2 cells incubated with testosterone, indicating testosterone can recruit mesenchymal pluripotent stem cells into the myogenic lineage in an AR-dependent manner (13). These data suggest testosterone-mediated changes in muscle AR content may be linked to the observed increase in post-exercise myogenic signaling (i.e., MyoD expression) in TEST versus PLA.

Observed changes in MyoD and markers of muscle protein breakdown in TEST versus PLA may be linked to upstream activity of the TWEAK receptor Fn14, a potent regulator a skeletal muscle mass. We observed lower Fn14 expression in TEST versus PLA at Base following the ED phase. Levels of Fn14 are generally low in healthy tissues and therefore the induction of Fn14 expression is tied to TWEAK/Fn14 pathway activity (33). Heightened activation of this pathway in TWEAK-treated myoblasts has been shown to impair myogenesis by decreasing gene expression and protein levels of MyoD and myogenin (34, 35). TWEAK-mediated activation of Fn14 signaling in cultured myotubes has also been shown to increase MuRF1 and MAFbx expression (36). These data suggest that the greater Fn14 expression

observed in PLA versus TEST in this study may be linked to the lower MyoD gene expression and greater levels of MAFbx and MuRF1 in these individuals. Interestingly, lower Fn14 expression in TEST versus PLA may be regulated by AR activity. Yin et al (37) reported an inverse relationship between Fn14 expression and AR signaling output (i.e., mRNA signature of AR target genes) in a microarray dataset composed of 131 primary and 19 metastatic prostate cancer samples. Predicting androgen response elements (ARE) in Fn14 promoter regions and subsequent analyses revealed that AR binding to the Fn14 enhancer decreased its expression (37). While it is not completely clear if these findings translate to muscle cells, the inverse association between Fn14 and AR observed in the current study support a similar mechanism of action human skeletal muscle. Therefore it is possible that AR-induced attenuation of Fn14 expression may mediate the increase in MyoD and attenuation of MAFbx and MuRF1 with testosterone supplementation during ED. The idea that AR and Fn14 regulate myogenic and proteolytic pathways to mediate the anabolic effect of exogenous testosterone during energy deficit is supported by the association between AR and change in leg lean mass ($r = .540$, $p = 0.02$) as well as Fn14 and change in leg lean mass ($r= -.455$, $p = 0.058$).

The findings of this study should be interpreted in the context of a few limitations. While mTOR-mediated anabolic signaling was not different between groups, we cannot confirm whether rates of muscle protein synthesis (MPS) were also unaffected by the testosterone intervention given the lack of stable-isotope derived measures of synthetic rates. Future work is needed to further elucidate the effect of testosterone supplementation on MPS during energy deficit. We acknowledge that our selection of the top (TEST) and bottom (PLA) 10 participants from the parent study with respect to changes in leg lean mass and total testosterone may also be considered a limitation. As discussed previously, the loss of lean mass and decrease in total

testosterone induced by energy deficit in the placebo group was less than anticipated (10). In fact, there were several individuals whose lean mass or total testosterone did not change following the 28-day energy deficit. Nonetheless, stratifying participants based on the change in total testosterone and leg lean mass allowed us to exclude these individuals to better characterize intracellular signaling and gene expression mediating the hypertrophic effect of supplemental testosterone during energy deficit.

The current study presents novel information about the molecular impacts of supplemental testosterone during a severe diet- and exercise-induced energy deficit. We report that testosterone supplementation during energy deficit upregulates myogenesis and attenuates ubiquitin proteasome-mediated proteolysis after exercise and feeding. mTOR pathway activation was not different between groups suggesting a negligible role of this signaling pathway in mediating the hypertrophic effect of testosterone supplementation during energy deficit. An increase in AR total protein content and decrease in Fn14 gene expression in TEST following ED, and the association of these markers with change in leg lean mass suggests signaling through these pathways mediates the effect of testosterone on downstream myogenic and proteolytic activity. Whether these findings can be effectively translated into androgen-related therapies for mitigating muscle losses during situations of extreme operational stress or clinical scenarios specific to muscle wasting remains to be determined.

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CHAPTER 6

Summary and Recommendations

Muscle atrophy occurs with reduced loading and neural activation of muscle after musculoskeletal injury (i.e., disuse atrophy), and during energy deficit resulting from inadequate caloric intake. Manipulating dietary protein or administering exogenous testosterone under these muscle wasting conditions may protect muscle mass and/or function by increasing muscle protein synthesis (MPS), attenuating muscle protein breakdown (MPS), and enhancing muscle regenerative capacity either directly or through upstream modulation of inflammatory signaling. Multiple investigations were conducted to assess the influence of dietary protein and testosterone supplementation on the regulation of muscle mass with disuse after injury and during energy deficit.

Results from Study 1 suggest higher protein intakes before ACL reconstruction surgery do not influence perioperative markers of inflammation and myogenesis. This was likely limited by the small sample size (n=5), however, given the large effect sizes ($d > 1.0$) and post hoc power analyses for several parameters that revealed a sample size of 10 to 18 subjects would be needed for group differences to reach statistical significance. Future investigations with larger sample sizes are needed to further elucidate whether manipulating dietary protein intake can influence inflammation and muscle regenerative capacity assessed at the time of surgery. Interestingly, a secondary analysis of a subset of subjects revealed several markers of inflammation and myogenesis at the time of surgery were associated with postoperative thigh circumference and quadriceps strength during postoperative rehabilitation. While we acknowledge that the small sample size (n=4) and correlative nature of this analysis are limitations, these findings provide intriguing preliminary evidence to suggest perioperative

muscle inflammation and regenerative capacity are associated with postoperative muscle recovery outcomes.

The case study included in Chapter 4 provides important insight into the role of dietary protein intake in regulating skeletal muscle mass during disuse following musculoskeletal injury. The patient's gains in injured leg thigh circumference following ACL reconstruction suggests that consuming ~2.0 g protein·kg^{-1}·d^{-1} may have benefited recovery by attenuating disuse atrophy and potentiating muscle hypertrophy during postoperative rehabilitation. The progressive increase in thigh circumference and quadriceps strength in the non-injured leg also indicates that physical rehabilitation is analogous to resistance exercise in promoting gains in muscle mass and strength, particularly when combined with a higher protein diet. Observations for this case study must be cautiously interpreted given the use of thigh circumference to estimate changes in muscle mass, as this measurement cannot differentiate the individual contributions of muscle mass and subcutaneous fat to total thigh size. Future work with larger populations is warranted to further understand how protein intake at the higher end of clinical recommendations protects muscle mass post-injury, and should use DXA, MRI, or CT scans to evaluate changes in muscle volume or cross sectional area.

Study 2 presents novel information about the molecular impacts of supplemental testosterone during a severe diet- and exercise-induced energy deficit. Findings suggest that exogenous testosterone administration during energy deficit attenuates proteolytic gene expression and enhances myogenesis after exercise and feeding. It also appears that AR-induced attenuation of Fn14 expression may regulate the observed changes in myogenesis and proteolysis to mediate the anabolic effect of exogenous testosterone during energy deficit. As discussed, stratifying participants based on changes in total testosterone and leg lean mass was necessary

given that there were several participants whose leg lean mass or total testosterone did not change during the energy deficit. While this may be considered a limitation, this method of analysis allowed us to exclude these individuals to better characterize intracellular signaling mediating the hypertrophic effect of supplemental testosterone during energy deficit. Future work inducing greater changes in testosterone status and lean mass during energy deficit by administering lower doses of testosterone or implementing greater stressors is needed to further elucidate the effects of testosterone supplementation on intracellular signaling regulating muscle mass during energy deficit.

In total, findings from Study 1 and Study 2 indicate integration of intramuscular inflammation, dietary protein intake, and testosterone-mediated intracellular signaling in the regulation of muscle mass during conditions of muscle wasting. This work is important in translating both protein-based diet interventions and supplemental testosterone therapies to the recovery from musculoskeletal injury and situations of extreme operational stress, respectively, in an effort to protect muscle mass and/or function during these muscle wasting conditions.

APPENDIX I

Orthopedic Associates of Hartford
Return-to-Sport after ACL Reconstruction Protocol

* This protocol is a time and criterion-based protocol, with a goal of maximizing healing and strength recovery, while minimizing risk of re-injury, so the athlete can return, as quickly and safely as possible, to athletic activities at the pace that is specific for each individual.

Immediately Post-Operative Phase:
(Week 1, approx. days 1-7 post-operatively)

Goals of this phase are:

- Restore full passive knee extension, and gradually ↑ knee flexion to 90°
- Diminish joint swelling and pain
- Restore patellar mobility
- Re-establish quadriceps control- active quadriceps contraction with superior patellar glide
- Improve ambulation to FWB/ ↓ assistive device use

Physical Therapy Interventions: Perform/increase number and duration of exercises as tolerated

- **Gait Training:**
    - Patient is WBAT in full locked extension brace with bilateral crutches, patient is provided instruction, demonstration and given verbal and tactile cues as appropriate to ensure the crutches are safe and are of the correct height for the should and hand grips
- **Exercises:**
    - Ankle Pumps to ↓ swelling/edema
    - Ankle resistance band open chain exercises- all 4 directions
    - Patellar mobilization in all directions
    - Quadriceps and Gluteal Isometric Setting
    - Hamstring Stretches- hamstring re-lengthening- **Gentle if H/S graft used**
    - Straight Leg Raises (flexion, abduction, adduction)
    - Gastrocnemius Stretching + Manual Release for re-lengthening + Knee /
    - Active and Passive Knee Flexion exercises to tolerance, not pushed- (to 90° by days 5-7)
    - Wall Heel Slides (supine with foot on wall/window) for increasing knee flexion, or seated AAROM
    - Gentle overpressure into full extension (PT or pt. actively)
    - Weight Bearing: weight shifts in full extension brace
    - Standing Hamstring Concentric & Eccentric- 90°⇔40° (no weight)
- **NMES:** Russian E-stim 2,500Hz with other appropriate settings):
    - Can and should be used, especially if contraction deficit is present, during active muscle exercises to enhance muscle contraction

- CP with full Extension, +/or Kinesiology Tape (basket-weave technique), dry needling technique, (control swelling/edema)
- **Home Exercises Program**- See HEP for Immediately Post-op Phase

Early Post-Operative Phase:
(~ 2 – 4 weeks Post-Op)

Criteria for entering this phase is:
1- Quadriceps voluntary initiation with quad set and SLR
2- Full passive knee extension
3- Knee A + PROM of 0°-90°, with good patellar mobility
4- Minimal joint effusion
5- Independent ambulation

Goals of this phase are:
- To maintain full passive and active knee extension
- To gradually increase knee flexion to 110°-120°
- To diminish swelling and pain
- Increase muscular training
- Restore proprioception
- Maintain patellar mobility
- Use a semi-recumbent cycle or NUSTEP without difficulty
- Perform a SLR with no extension lag
- Reciprocal stair climbing
- KOS-ADL Score of >65%

Physical Therapy Interventions:
- **Gait Training:** WBAT, with a goal of discontinuing use of crutches by day 8-14 post-op- continue locked brace until voluntary quad control is demonstrated, ***May be subject to change by MD**
- **Exercises:** (**continue/progress all exercises from previous phase)**
    - Continue NMES with quadriceps exercises, if needed
        - ~ 4 weeks: Quadriceps isometrics multiple angles (90°, 70°, 50°)
    - SLR in all 4 planes- Hip Abd performed with ~10°-15° of Hip Extension, possible progression- 1# weight (***Proximal loading above knee joint**)
    - ~ 4 weeks: OKC –Knee Extension 90° to 40°, with or without NMES
    - Mini Squats 0°-30° DL (BW only) Progress to unstable surface such as tilt board (Med./Lat. & Ant./Post.) or foam with 3-5 second holds ***Forward trunk tilt- recruit H/S & unload ACL**, Wall Squats to 40°
    - Overpressure into Full Knee Extension, Prone Knee Hangs if it is lacking
    - Hamstring Curls **(* Only for B-T-B Graft)**- **~8 weeks for H/S Graft**
    - PROM 0° to 110°-120°
    - Weight Shifts- Medial/Lateral & Diagonal Patterns, Balance Weight Shifting- balance board, BOSU

    - Forward & Lateral Mini Lunges (30°-40° of flexion)- **if patient has good quadriceps control**
    - Step-Ups (pain free range)- Front & Lateral- *** Low Step initially**
    - Lateral Step Over: Cones, Hurdles- ***Instruct to raise knee to level of hip**
    - DL Leg Press- ***Sub-Maximal**
    - Stationary cycle (if ROM permits)/ rocking on LE cycle **(not forced)**
- **Manual Treatments:**
    - Patellar Mobilization (If flexion is limited), Scar Mobs, (If skin is healed)
    - Continue to control swelling: Ice, elevation if needed

Intermediate Post-Operative Phase:

(~4-8 weeks Post-Op)

Criteria for entering this phase:

1- P & AROM knee flexion to ≥ 110°
2- Quadriceps & Hamstring strength to >60% of non-injured leg (dynamometry)
3- Minimal to no effusion
4- No joint line or patellofemoral pain
5- Mild laxity on clinical examination, or +1 mm or less with the KT-2000 test
    a. Week 4 & 6: KT-2000 test at 20 & 30 lbs anterior and posterior

Goals of this phase are:

- Quadriceps strength to ≥75% of the non-injured leg, H/S ≥75% non-injured leg
- Restore knee ROM to 0°-125°, or knee ROM to within 10°-15° of non-injured leg
- Improve lower extremity strength
- Enhance proprioception, balance, and neuromuscular control
- Improve muscular endurance
- Restore limb confidence and function
- KOS-ADL score or >75%
- Get an Post-Op score for IKDC or KOOS (age appropriate version)

Physical Therapy Interventions:

- **Gait Training:** Unlocked brace, discontinued use if sufficient quad strength is present. Retrain with normal walking gait pattern
- **Exercises:** (**Continue/progress all from previous phases as tol./as needed)**
    - OKC Knee extensions: 90°-40°
    - Front & Lateral step down exercise
    - Heel Raises (raising up onto toes)
    - Progress lateral stepping exercise
    - Mini Squats with forward trunk tilt (to decrease ACL strain + recruit H/S)- in Patient's ROM and Tolerance, not lower than 40°-50° of knee flexion
    - Perturbation Training – DL-> progress to →SL Balance when appropriate- Can also use uneven surfaces and external perturbations to progress (DL & SL)- Tilt Board (stabilized to level position initially), BOSU®, Airex®- progress with ball

toss & catch/reaching (UE & LE), external tapping on hips and trunk on any surface- ***With knee slightly flexed**

- Eccentric Exercises: (for H/S & Quad)- Decline Squat, DL+SL Squat, etc.
- Pool Exercises if available: forward & backward walk/run **(*forward at week ≥6)**, hip & leg exercises- **slow speeds of mvmt. (Graft protection)**
- Begin testing/training for proprioception on the Biodex® Stability System (or other system)- ***if available, or**: BESS Test, Functional Reach Test, Tandem Walking tests- **See attached sheets**
- Progress Stationary Cycle and Walking- min of 10 minutes, increasing 10min/wk (walk)
- Core Stabilization/Strengthening- Bridging (Progress with Band: Abd→ unstable surface→ hamstring curl w/ball), SL Bridge (knee /, Valslide®)

- **Manual Treatments:**
  - Continue patellar and scar mobilization if needed
  - Tibiofemoral mobilization with rotation for ROM if needed

Late Post-Operative Phase:
(~8-12 weeks Post-OP)

Criteria for entering this phase:

1- Active knee ROM 0°- ≥125°
2- Quadriceps Strength & Girth ≥75% of non-injured leg (dynamometry & tape measure), Hamstring Strength ≥75% of non-injured leg (dynamometry)

Goals for this phase:

- Restore full knee ROM
- Continue to improve lower extremity strength, balance, proprioception, neuromuscular control, muscular endurance
- Knee effusion to trace or less
- Quadriceps strength to >80 %, Hamstring Strength >80% of the non-injured leg
- Normal gait pattern
- Minimal laxity on clinical exam, or 2mm or less on the KT-2000 test
- Get a baseline FMS® and Y-Balance™ score at ≥**8** weeks post-op- when appropriate- Begin with the SFMA (no pain- can proceed to other tests)
- Patient is tested with the Landing Error Scoring System (LESS): **See Attached Sheets: - Test at Patient's individual max vertical jump height, not standard measure on the LESS- may be too high or too low for accurate individual jump assessment score, no sooner than 10 weeks post-op**
  - Excellent Score is </= 4
  - Good Score is >4 and </=5
  - Moderate Score is >5 and </=6
  - Poor Score is >6
- Perform hop testing at no sooner than **10** weeks post-op: **See Attached Sheets**
  - 2 practice trials on each leg, then 2 timed or measured trials an each leg; measure and average to compare injured to non-injured legs

    - 1- Single-leg hop for distance
    - 2- Triple hop for distance
    - 3- Single-Leg Crossover triple hop
    - 4- 6-Metered timed hop

Physical Therapy Interventions:

- **Exercises: (<u>Continue/progress all exercises from previous phases as tolerance/as needed)</u>**
    - <u>Progress exercises in intensity & duration, Advance core stab. exercises</u>
    - <u>Continue Perturbation Training-</u> Internal & External Cues
    - <u>Dynamic/Plyometric Leg Press</u> (<u>Begin no sooner than 8 Weeks</u>- To learn technique and control ground reaction forces, "land softly on toes with knees slightly flexed" for dissipation of force and to avoid hyperextension)
    - <u>Progress lateral stepping and lateral step-down exercises</u> with resistance bands on the distal femur creating a medial pull (bilateral with stepping, SL with lateral step-down)
    - <u>Begin walk→run protocol</u>, toward the end of phase, when patient is able to perform a controlled single leg squat (injured and non-injured legs) to 60° of knee flexion- **see attached protocol**
    - <u>Continue Eccentric Training:</u> (See sheet)

Early Activity Phase:

(~12-16 weeks Post-Op)

Criteria for entering this Phase:

1- Full knee AROM
2- Quadriceps Strength & Girth >80% of the non-injured leg, knee flexor-extensor ratio of 70% to 75%, Hamstring Strength >80% of the non-injured leg (Dynamometer)
3- No pain or effusion
4- No laxity/instability on clinical exam, or KT-2000 test of 2mm or less compared to the non-injured side
5- Hop Tests (80% of non-injured leg)
6- IKDC (use for concomitant injuries)- score of 80% or higher, or the KOOS (use with ACL alone) score of ≥80

Goals for this phase:

- Normalize lower extremity strength (<15% difference Left to Right)
- Increase muscle power and endurance, & begin selected skill drills
- Maintain/Gain Hamstring & Quadriceps Strength and Girth of 80% or greater
- Hop Tests to > 85% of the non-injured leg
- Continue to improve neuromuscular control
- KOS-sports score of > 70%
- A score of <u>≥14/21</u> on the FMS® Assessment Screen, with **No** 0/3= pain on any of the 7 fundamental movement patterns
- <u>No statistical asymmetries</u> and on the Y-Balance Assessment™
- Subjective Knee score (Cincinnati Knee Rating System) of 80 points of higher

Physical Therapy Interventions:

- **Exercises: (Continue/progress exercises from previous phases as tol./need.)**
    - Agility exercises- side shuffling, cariocas, zigzags- **when appropriate and cleared by MD**
    - Advanced Core & Hip Stab. exercises: Resisted Clam, Quadruped Reach w/Resistance, Lateral Squat Stepping, Side+ Prone Planks w/LE Lift, etc.
    - Continue/Progress Walk-to-Run Protocol
    - Begin Plyometric Training Protocol- when appropriate- landing screening, Hop Tests, and no increase in pt's symptoms- **See attached sheets**
    - CKC exercises may be progressed to 75°-90° of flexion
    - Continue Eccentric exercises: For all LE muscles-ex. Step-Down exercises

Functional Assessments:

- FMS® and Y-Balance Assessment™ **at beginning and end of this phase**
- Repeat the 4 Hop Tests from the previous Phase **at beginning and end of phase**

Core Testing:

- Segmental Multifidus Test
- Trunk Curl Up Test
- Double-Leg Lowering Test
- Side Bridge Test
- Prone Bridge Test
- Supine Single-Leg Bridge Test
- Extensor Endurance Test

Return to Activity Phase:

Criteria for entering this Phase:

1- Full ROM
2- A score of ≥14/21 on the FMS® Assessment Screen, with **No** 0/3= pain on any of the 7 fundamental movement patterns
3- No statistical asymmetries and on the Y-Balance Assessment™
4- Hop Tests (90% or higher compared to non-injured leg)
5- Limb Symmetry Index (LSI) of 90% or greater on hop tests
6- IKDC Score ≥ 85%, or the KOOS score of ≥85
7- KOS-Sports Score 90% or greater
8- Cincinnati Knee Rating System score of 290 points or higher
9- No change in knee laxity (clinical exam or </=2mm on KT 2000 test)
10- Isokinetic testing: (if available)
    i. Quadriceps (80% or greater) compared to non-injured leg
    ii. Hamstring (100%-110%) compared to non-injured leg
    iii. Hamstring-Quadriceps Ratio (70% or greater)

Goals or this Phase:

- Achieve maximal strength and endurance

- Normalize neuromuscular control
- Progress to skill training
- Gradually return to sport specific training

Physical Therapy Interventions:

- Exercises:

- Continue strengthening exercises
- Continue/Advance core training exercises
- Continue Neuromuscular control exercises
- Continue plyometric exercises
- Progress running program
- Begin agility and skill training exercises:
  - Continue cariocas, zigzags, side-shuffling
  - Begin sudden start and stops, figure-8's, 45° and 90° cutting drills, box jumps (progressing & varying heights up to 20cm)

Functional Assessment:

- Repeat 4 previous hop tests, plus Hop-to-Stop test- **at the end of this phase**
- FMS® and Y-Balance Assessment™ **at the end of this phase**

APPENDIX II

Standardized Physical Rehabilitation Exercise Protocol

Initial Post-op Phase (~1 week Post-Op)

- **PROM Knee** 0°- 90° (as close to full extension as you can get the patient)
- **Manual Techniques**: tissue flexibility, □ swelling, ROM, Patellar Mobility, □ Pain
- **Gait Training:** with Bilateral Crutches in Locked Extension Brace
- **Exercises:**
    - Ankle Pumps
    - Ankle Resistance Band Exercises- All 4 Directions
    - Quadriceps and Gluteal Set Exercises
    - SLR: Abduction, Flexion **with Brace**
    - Calf Stretches with strap
    - Heel/Wall Slides for knee flexion
    - Weight Shifting in Locked Extension Brace

Early Post-Op Phase (~ 2-4 weeks Post-Op)

- **Continue Manual Techniques** as needed, add scar mobilization when incision is healed
- **ROM**: 0°-120°
- **Gait Training:** WBAT D/C Crutches as tol. (**MD Decision**, ~8-14 days Post-Op)
- **Exercises:**
    - SLR: all 4 Planes (Hip Abduction performed with 10°-15 of hip extension)
    - NMES: (~4 weeks Post-Op)
        - OKC Knee Extension: (90°-40°)- *** No Weight**
        - Quadriceps Isometrics: (90° and 60°)- ***No Weight**
    - Mini Squats: DL (0°-30°) **Body Weight Only**- with forward trunk tilt (to □ ACL load)
    - Front and Lateral Step-Ups: (low Step- in pain-free range)
    - DL Press **(*Sub-Maximal)**
    - Lateral Step Overs: (Instruct patient to raise knee to the level of the hip)
    - Stationary Cycle **(Not Forced):** Rocking back and forth if full revolution cannot be achieved
    - *** See full Protocol for Phase Progress Criteria**

Intermediate Post-Op Phase (~4-8 weeks Post-Op)

- **Continue Manual Treatments** as needed
- **ROM:** 0°-125°, (or within 10°-15° of uninjured knee)
- **Gait Training:** Brace D/C'd **by MD** during this phase- Normalize the Gait Pattern
- **Exercises:**
    - NMES: OKC Knee Extension (90°- 40°) & Isometrics (90°, 60°)

- Heel Raises (DL & SL)
- Mini Squats with Forward Trunk Tilt **(no lower than 45°-50° Knee Flexion)**
- Front and Lateral Step Downs
- Continue Quadriceps and Hamstring Stretches
- Side-Lying Clam Exercise (progress with resistance band as tol.)
- Continue SLR Hip Abduction (with ~10°-15° Hip Extension)
- Standing Hamstring Curls: **(~8 weeks Post-Op)**- Concentric and Eccentric
- Bridges: (~8 weeks Post-Op) DL
- DL & SL Leg Press: (□'ing resistance)
    - **Progress from DL to SL as tolerated**
- *** See full Protocol for Phase Progress Criteria**

Late Post-Op Phase (~8-12 weeks Post-Op)

- **Continue Manual Treatments** as needed
- **ROM:** Should be equal to uninjured knee
- **Exercises:**
    - DL + SL Squats: (Internal and External Cues)
        - Tape vertically on knees and floor about shoulder width apart, Patient must keep the tape pieces lined up
        - Resistance band:
            - **To Assist Equal Weight-Bearing-** Band at waist tied off laterally on side of injury to cue to load injured LE
            - **Knee Valgus-** Band just about injured knee pulling medially, so patient has to resists going into Valgus
            - **Trunk Rotation**- Band around ipsilateral shoulder to injury, then it goes around the back and around the contralateral side waist and is tied off anterior to the patient. As patient goes into the squat they must resist trunk rotation.
    - SL Anterior Reaches: (with or without Valslide®)
    - Progress Forward and Lateral Stepping Exercises: (□ step height)
    - Bridges with Knee Extension: (DL/SL), and with ball H/S curl
    - Anterior and Lateral Lunges: (tolerable range) with Knee Valgus/Varus control
    - Dynamic Stretches:
        - Toe + Heel Walks, Knee Hugs, Butt Kicks, Toe Swipes, Monster Walks, Fig-4 Squat walk
    - Perturbation Training: (~10 Weeks Post-Op)- SLS on Foam/Wobble board/Balance Disc
        - Progress to include ball toss/catch, Bird Dips/Dead Lifts **(0#/ light weight initially)**
    - *** See full Protocol for Phase Progress Criteria**

Early Activity Phase (~12-16 weeks Post-Op)

- Exercises:
    - Continue Bridge Exercises
    - Progress Clam Exercise: with Resistance Band
    - Continue DL + SL Squats:
        - Progress Internal (Surface, Tape) and External (Bands, Ball Toss, Manual Touches) Perturbations
    - Lateral and Forward Squat Walks with Resistance Band:
    - Quadruped UE+ LE Reaches: with Resistance Band
    - Side **and** Prone Planks: (10 Seconds holds)
        - **Progress with Leg Raises**
    - Dynamic Stretches:
        - All Previous Stretches, **plus:** Ankle Grab 1-hand floor touch, lift added to Fig-4 Squat, High Knee Jog, Inverted Hamstring Stretch, Anterior Lunge with Upper Body Extension, Lateral Lunge, and Inchworm Stretch
    - Dynamic/Plyo Leg Press/Jumps
    - Begin Walk-To-Run Protocol: **Patient Must be able to demonstrate Controlled SL Squats to 60° of Knee flexion**
    - *** See full Protocol for Phase Progress Criteria**